The madness of microbiome: Attempting to find consensus “best practice” for 16S microbiome studies

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Running Head: Consensus "best practice" for 16S studies

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Abstract

The development and continuous improvement of high-throughput sequencing platforms has stimulated interest in the study of complex microbial communities. Currently, the most popular sequencing approach to study microbial community composition and dynamics is targeted 16S rRNA gene metabarcoding. To prepare samples for sequencing, there are a variety of processing steps, each with the potential to introduce bias at the data analysis stage. In this short review, key information from the literature pertaining to each processing step is described and consequently, general recommendations for future 16S rRNA gene metabarcoding experiments are made.

Introduction

In recent years, the emergence of high-throughput sequencing platforms has revolutionised the study of complex microbial communities. Most commonly, marker genes (e.g. 16S rRNA and 18S rRNA genes) are amplified and sequenced, providing both qualitative and quantitative (i.e. relative abundance) data. However, the variety of methodologies which can be used to carry out marker gene analysis can be overwhelming. Each methodological stage, from sampling to data analysis, can introduce biases, and such biases can skew datasets by introducing changes in the relative abundances observed and can affect the perception of community diversity. This short review includes key information from current literature on sample collection, sample storage and processing, and sequencing and data analysis; specifically for the study of bacterial communities using 16S rRNA gene metabarcoding. By collating fundamental research from each of these areas, we aim to try to ensure that scientists entering this field are better informed to make decisions on experimental design for 16S rRNA gene sequencing studies.

Sample collection

Sampling method is obviously dependant on sample type and as such, the factors which may introduce bias will also vary between different types of microbiome studies. Clearly, study-specific concerns cannot be entirely covered in this review. However, the overarching factors which should be taken into account will be briefly covered in this section.

Firstly, it is important to consider the proposed sampling site. Bacterial community composition varies even within a specific environment, for example at different sites within the gastrointestinal tract (1), the respiratory tract (2) and at different soil depths (3, 4). Since the magnitude of inter-individual variation is very much dependant on sampling site (5), this can have implications for experimental design, specifically when considering the number of subjects and the number of samples to be taken.

Secondly, there are conflicting results in the literature with regards to the variation introduced by different sample collection methodologies. For example, there have been attempts to replace invasive sampling with less invasive methods; however, significant differences have been found in microbial populations when comparing swab and biopsy samples from human intestines (6), when comparing breath condensate and lung brushings (7) and when comparing rumen fluid samples obtained via oral stomach tubing and a fistula (8). However, other work contradicts these findings, with two studies showing no statistically significant differences when studying the rumen microbiota in cattle using a variety of sampling methods (9, 10). Additionally, no significant differences were evident in microbial composition when comparing sino-nasal swabs and biopsy samples (11) and rectal swabs and stool samples (12). This kind of conflict in the literature is not uncommon, which leads to a lack of consensus and standardisation.

A final consideration is whether samples should be homogenised, which appears to be most critical in studies on gut contents (8, 13) and on soil (14), since varying microbial

compositions have been observed in different stool fractions and in soils with varying particle size.

Although the literature is generally conflicting with regards to sampling methodology, it is important to consider that comparing data obtained using different approaches should be avoided.

Sample storage

There is conflicting evidence on whether different storage conditions alone can have an impact on microbial community studies (15–18). It is often not practical to extract DNA from fresh samples, therefore samples are generally stored for varying durations prior to DNA extraction. Conventionally, it is assumed that rapid freezing to -80^{o}C is best practice (18, 19) but this is not feasible for all study designs, for example, at remote sites where low temperature storage is unavailable (20). Several studies have been carried out to assess the effects of storage conditions on study findings, which will be summarised in this section.

Fresh versus frozen samples

A couple of studies showed that freezing samples appeared to cause an increase in the Firmicutes to Bacteroidetes ratio in comparison with fresh samples (15, 19). Conversely, in a study by Fouhy *et al*, the only bacterial groups differentially expressed between fresh and snap frozen faecal samples were the *Faecalibacterium* and *Leuconostoc* genera, with no significant differences being evident at phylum or family levels (18). No significant effects on microbial composition or diversity were observed in faecal samples refrigerated for 24 hours (21) or 72 hours (20) prior to DNA extraction.

The impact of storage duration has also been explored in various studies. Lauber *et al* stored soil, faeces and skin samples at various temperatures and found that storage duration had no significant impact on overall bacterial community structure or diversity (17). In samples which were stored at -80^{o}C for 2 years, a small number of changes in the microbial communities were observed with increased abundances of lactobacilli and bacilli, and a reduction in the total number of operational taxonomic units (OTUs) (for a definition of OTUs, please see section entitled "operational taxonomic unit picking methods").

When considering the data presented in the literature, generally processing fresh samples is the best approach but when this is not possible, samples should be frozen for an unequal amount of time and processed in one batch or frozen for an equal amount of time and processed in multiple batches. The decision on how to proceed will be dependent on the duration of the sample collection phase and on study design, but regardless of processing method, storage duration and DNA extraction batch should be recorded to enable this to be taken into account during analysis.

Use of cryoprotectant

McKain *et al* explored the effects of using a cryoprotectant (i.e. glycerol/phosphate buffered saline) to store ruminal digesta samples and found that freezing samples without cryoprotectant caused a significant loss in Bacteroidetes when measuring 16S rRNA gene copy number by quantitative PCR (15). The authors consequently suggest that simply storing samples without a cryoprotectant and carrying out DNA extraction at a later date would impact downstream results when considering archaeal and bacterial community composition. Choo *et al* explored the effects of using several common preservative buffers (i.e. RNAlater, OMNIgene.GUT and Tris-EDTA) relative to samples stored dry at -80^{o}C on faecal

119 microbiota composition (20). Samples stored in the OMNIgene.GUT buffer diverged the
120 least from the samples stored dry at -80^{o}C and the results obtained from the samples stored in
121 Tris-EDTA diverged the most, with associated changes in relative abundances of biologically
122 important bacterial species such as *Escherichia-Shigella*, *Citrobacter* and *Enterobacter*.
123 Additionally, RNAlater has previously been shown to be unsuitable for storage of samples
124 subject to microbial community analysis, with samples stored in RNAlater being the least
125 similar to fresh samples and samples immediately frozen at -80^{o}C (22, 23).

126 **Consequently, when considering the use of a cryoprotectant for storage, it is important**
127 **to ensure that all samples are stored in the same manner.**

128

129 **DNA extraction**

130 During DNA extraction, it is important to consider that some microbial cells may be more
131 resistant to lysis, such as bacterial endospores (24) and Gram-positive bacteria, which will
132 have an impact on DNA extraction efficiency. The presence of inhibitors has also been found
133 to directly impact DNA extraction efficiency (e.g. debris in environmental samples, organic
134 matter in soil and faeces) and can affect the efficiency of PCR downstream (reviewed in
135 detail by Schrader *et al* (25)). Common inhibitors include inorganic material (e.g. calcium
136 ions), with the majority of inhibitors being organic matter such as humic acid, bile salts and
137 polysaccharides. These issues will vary according to sample type, therefore, matrix-specific
138 DNA extraction protocols should be optimised as part of a 16S rRNA gene metabarcoding
139 experiment.

140 Besides phenol-chloroform DNA extraction methods, there are many commercial extraction
141 kits available which incorporate mechanical and/or chemical/enzymatic lysis steps.
142 Numerous authors have demonstrated that the abundances of specific bacterial groups vary

143 when comparing different DNA extraction methodologies (8, 26–31). Specifically, variations
144 in DNA yield and quality are obtained which can lead to different results in downstream
145 analyses (28).

146 One key DNA extraction step which can introduce bias is the presence or absence of a
147 mechanical lysis step. The inclusion of a bead-beating step has been linked to a higher DNA
148 yield (8, 29, 32), higher bacterial diversity (29, 32) and more efficient extraction of DNA
149 from Gram-positive and spore-forming bacteria (29, 33, 34). Consequently, some authors
150 suggest that samples subject to different DNA extraction methods are not comparable (8, 28,
151 35).

152 **Ultimately, the best approach is to utilise a method which extracts the highest yield and**
153 **quality of DNA as possible without biasing the method towards particular bacterial**
154 **taxa. To achieve this, inclusion of a bead beating step and prior optimisation of the DNA**
155 **extraction method to ensure optimal DNA yield and quality is recommended prior to**
156 **carrying out 16S rRNA gene sequencing.**

157

158 **Sequencing strategy**

159 **Library preparation**

160 Since the entire 16S rRNA gene cannot be sequenced using short-read second-generation
161 sequencing platforms, a short region of the gene must be selected for PCR amplification and
162 sequencing. There is currently no consensus on the most appropriate hypervariable region(s)
163 and several studies have been carried out to determine the advantages and disadvantages of
164 each. Importantly, the choice of hypervariable region(s) and the design of the "universal"
165 PCR primers have an effect on phylogenetic resolution (36–40). Indeed, no primer set is
166 truly universal, with some commonly used 16S rRNA gene primers proving ineffective at

167 amplifying biologically relevant bacteria (34, 41). Fouhy *et al* explored the effects of primer
168 choice (as well as DNA extraction and sequencing platform) on microbial composition data
169 using a mock bacterial community and three primer sets (42), with differences in relative
170 abundances and richness being observed.

171 Further biases can be introduced during PCR amplification due to the presence of PCR
172 inhibitors (described in the DNA extraction section), with the number of PCR cycles and the
173 use of a high-fidelity polymerase (43) also having an impact on results. The formation of
174 chimeras occurs in later PCR cycles when the highest concentration of incompletely extended
175 primers compete with the original primers. Consequently, the potential for chimera
176 formation can be reduced by lowering the number of PCR cycles (44). Previous work found
177 that bacterial richness increased as the PCR cycle number increased (45, 46), but that cycle
178 number had no significant effect on community structure (46). A lower number of PCR
179 artefacts were found when using a high-fidelity polymerase compared to a standard
180 polymerase (43). The use of different polymerases has also been found to significantly affect
181 PCR efficiencies for particular bacterial groups and the overall bacterial community
182 structures (46). Finally, the quantity of input DNA into a PCR reaction has also been found
183 to have a significant effect on observed bacterial community structure (31).

184 **In summary, there is not a "gold standard" hypervariable region for 16S sequencing**
185 **but it is important to consider that PCR reagents and PCR conditions should be**
186 **optimised and kept consistent across a study.**

187

188 **Sequencing platforms**

189 D'Amore *et al* have studied the choice of sequencing platform most recently (47) and we
190 would refer the reader to that manuscript for a more in depth analysis. Illumina technology

191 (primarily the MiSeq) has become the most common sequencing platform for 16S rRNA
192 gene metabarcoding. This is because the MiSeq, in general, produces the most accurate,
193 longest reads and has a much higher throughput than the other platforms, which enables more
194 samples to be sequenced at higher depth or cheaper cost. Indeed, whilst D'Amore *et al*
195 caution that the choice of sequencer depends on the question being asked, they note that the
196 MiSeq is likely to be the platform of choice in most cases. The Roche 454 sequencer was, for
197 a long time, the platform most used for 16S studies. The potential longer reads of this
198 technology have some advantages; however, it is now no longer available as Roche retired
199 the product in 2013. The 454 unfortunately suffered from an elevated error rate due to mis-
200 calling of homopolymers. The Ion Torrent and Ion Proton platforms are often available at
201 low capital cost, and produce data more quickly than the MiSeq. However, the lower
202 throughput and higher error rates mean that many researchers prefer to select the MiSeq.
203 Whilst Illumina offers the highest quality data, there are some reported problems with the
204 platform. Illumina error rates are often thought to be around 0.01%, however Kozich *et al*
205 showed the actual error rates can be as high as 10%, and recommend a complete overlap of
206 250 bp reads to correct for this (48). D'Amore *et al* similarly showed library-dependent error
207 rates in either read 1 or read 2 (but not the overlap) in MiSeq data, albeit at a lower rate (2-
208 3%) (47). An improvement has been suggested to this involving a heterogeneity spacer that
209 improves sequence diversity in the library (49).

210 PacBio and Oxford Nanopore technologies are able to sequence the full length of the 16S
211 gene, which is of course very powerful. However, again error rates are an issue, in the range
212 of 5-15% for both technologies, which can cause subsequent errors in downstream analysis.
213 Despite the high error rate of long-read single molecule sequencing systems (50–52), studies
214 are beginning to appear to show their utility for 16S rRNA gene sequencing (53–56). For
215 example, Schloss *et al* were able to reduce the observed error rate for the V1–V9 region from

0.69 to 0.027% for PacBio data, which is comparable to Illumina, 454 and Ion Torrent systems (54). One of the drawbacks of the PacBio technology is throughput, which means that the number of samples that can be run on the platform simultaneously and at reasonable cost is much lower than the MiSeq.

When planning a 16S sequencing study, three key considerations are the quality of sequence data, the cost of sequencing and the length of generated reads, as detailed already in this section. A final factor is the number of samples which can be analysed per sequencing run. When considering Illumina platforms specifically, it is possible to use multiplexing strategies by implementation of unique single-indexed (57) or dual-indexed (48) (or barcoded) primers for library preparation. If the number of samples per run is increased, this is associated with a lower coverage (or number of sequences generated) per sample. If the coverage per sample is too low, then the diversity of the microbial community being studied is likely to be under-represented, as rarer members of the community are less likely to be detected. Therefore, guidance on the number of samples to be included per run should be obtained from small pilot studies (and observation of the resultant rarefaction curves) or published literature. In larger studies, more than one sequencing run may be required and Caporaso *et al* showed that data were highly reproducible across sequencing lanes (57).

The appropriate sequencing platform should be selected based upon the aims of the experiment and the error rates associated with the available platforms. Another key consideration is sequencing coverage and its relation to the number of samples to be run. When studying core members of a microbial community, lowering the amount of coverage by increasing the number of samples in a sequencing run may be an effective way to decrease costs. However, if rarer members of a community are of interest lower sample numbers leading to increase coverage may be more appropriate.

240

241 **Mock bacterial communities**

242 As part of 16S microbiome studies, it is useful to include a mock community control
243 composed of pre-determined ratios of DNA from a mixture of bacterial species. This not
244 only allows the quantification of sequencing error (58) but also allows bias introduced during
245 the sampling and library preparation processes to be identified (42, 47, 59, 60). For example,
246 a mock community containing bacterial taxonomies which are of specific interest to the
247 research group can be used to calculate whether these taxonomies are likely to be over or
248 under represented in samples. Similar to mock communities, spike-in standards can also be
249 used to analyse bias and the reproducibility of methodologies (61). However, unlike mock
250 communities, these standards are added directly to samples and therefore quality control can
251 be performed on a per sample basis. However, there is a risk of crossover between the 16S
252 rRNA gene sequences contained in the standards and those which may be found in samples.
253 Consequently, care must be taken to select bacteria which are highly unlikely to occur in the
254 samples of interest (62, 63) or which have been designed *in silico* and are dissimilar to
255 sequences found in 16S databases (61).

256 There are a variety of sources which provide mock bacterial communities for use in research;
257 however some researchers choose to create their own mock communities in-house which
258 more accurately reflect bacteria of interest and scientific importance. Pre-prepared bacterial
259 communities are available in two different formats – DNA mock communities and whole cell
260 mock communities. The latter is useful for establishing the efficiency of the DNA extraction
261 step, whereas the former will only assess the efficiency of PCR, clean up, sequencing and
262 analysis steps. At the time of writing, mock communities are available from the American
263 Type Culture Collection (ATCC) and Zymo Research.

When planning a 16S study, the inclusion of a mock community is strongly encouraged.

Analysis strategy

Comparing pipelines

The analysis of large and complex 16S rRNA gene sequencing data sets requires the use of bioinformatic tools. There are many pipelines available to process and analyse 16S rRNA gene sequencing data, including the commonly used QIIME (64), MG-RAST (65), UPARSE (66) (URL: https://www.drive5.com/usearch/manual/uparse_pipeline.html) and mothur (67). These packages contain sets of tools which facilitate the complete analysis of 16S rRNA gene data, from quality control to operational taxonomic unit (OTU) clustering. Where they differ is predominantly in their accessibility to those with limited computational knowledge and in the availability of documentation.

Nilakanta *et al* compared seven different packages (mothur, QIIME, WATERS, RDPipeline, VAMPS, Genboree, and SnoWMan) and concluded that while all of these packages provide effective pipelines for 16S rRNA gene analysis, the extensive documentation which accompanies mothur and QIIME provides them with an advantage over the other packages (68). Plummer and Twin analysed a single data set using QIIME, mothur and MG-RAST and found that there were few differences in the results when considering taxonomic classification and diversity (69). However, there were differences in the ease of use of each of these packages and the time required for analysis, with QIIME being the quickest analysis package (approximately 1 hour) and MG-RAST being the slowest (approximately 2 days, due to the need for manual quality control to remove multiple annotations of reads). The authors do state that although MG-RAST is the slowest analysis method, it is perhaps the most suitable package for users with no command line experience.

Ultimately, the choice of analysis package will be made on the basis of the user's level of experience in bioinformatics and on the available resources at the user's host institution.

Quality control, alignment and taxonomic assignment

It is essential to carry out quality filtering to remove DNA sequences which are of unexpected length, have long homopolymers, contain ambiguous bases or do not align to the correct 16S rRNA gene region. Critically, sequences should then be screened for chimeras, as the presence of chimeric sequences can affect the interpretation of the final dataset and could, for example, over-inflate perception of community diversity (70). A variety of tools have been developed to remove chimeric sequences such as UCHIME (66) and Chimera Slayer (70). By including a mock bacterial community in a sequencing run, since the true sequences in these are known, the number of chimeric sequences can be calculated (58).

Sequences should then be aligned to a reference alignment, or assigned to a suitable reference using a sequence classifier such as the RDP classifier which uses a naïve Bayesian approach based on 8-mers (71). Schloss showed that alignment quality can significantly impact diversity and can artificially inflate the number of bacterial OTUs, and advised against using alignments which do not take into account the secondary structure of the 16S gene (72). Of the three most commonly used alignments which are guided by secondary structure (i.e. greengenes (73), RDP (74) and SILVA (75)), the greengenes alignment was observed to be of poor quality, leading to significantly greater richness and diversity estimates.

Post-alignment, sequences and OTUs are assigned taxonomies based upon their similarity to training sets, most commonly constructed from the greengenes, RDP and SILVA databases. Errors within these databases, caused by sequencing/PCR errors (76) or by the incorrect

312 labelling of sequences (77), may lead to the misidentification of sequences. Another issue
313 when relying on databases for taxonomic assignment is their bias towards bacteria which are
314 clinically relevant in humans, meaning that researchers investigating non-human hosts or
315 environmental samples may struggle to assign taxonomy to their sequences. For example, in
316 a study of the honey bee gut microbiota, disagreement was found between the three databases
317 listed above upon carrying out taxonomic assignments (78). At genus level, the three
318 databases concurred in their assignments for only 13% of sequences. The classification of
319 sequences was improved by including bee-specific full length 16S rRNA gene sequences in
320 the training set, highlighting the need to include more representative sequences from a greater
321 number of habitats.

322 This has been highlighted by Werner *et al* who advised using the largest and most diverse
323 database possible (79). This group also found that trimming the reference sequences to the
324 primer region of interest improved classification depth. However, in a more extensively
325 studied environment such as the human intestine, Ritari *et al* found that making a
326 personalised reference database containing only bacterial species which were known to
327 inhabit that niche led to an increase in lower taxonomic level assignments, probably due to
328 less competition among sequences compared to large databases (80).

329

330 **Operational taxonomic unit picking methods**

331 Operational taxonomic units, or OTUs, are the common currency of 16S or marker gene
332 studies of microbiomes. The term was originally coined by Sokal and Sneath (81), and in its
333 more general usage refers simply to groups of organisms that are closely related. There are
334 two major methods for defining OTUs – reference-based and *de novo*. In reference-based
335 clustering, sequences from a community are clustered against a known reference database,
336 and in *de novo* clustering, the sequences are clustered according to pairwise distance

337 measures. Reference-based OTUs are sometimes referred to as "*phylotypes*" (82). As with
338 many areas of microbiome analysis, the evidence is mixed as to which of the two approaches
339 is best. It has been found that *de novo* methods perform better when considering the quality
340 of OTU assignments (83), with another study showing that de novo OTUs were unstable
341 (84). However, Westcott and Schloss argued that OTUs can be stable yet still incorrect, and
342 in particular showed that some reference-based techniques were sensitive to the order of
343 sequences in the database. Sul *et al* found that reference-based techniques produced similar
344 results to *de novo*, with the added benefit of low computational overheads and the ability to
345 compare datasets from different variable regions (85). Indeed, perhaps the major difference
346 between reference- and *de novo* based methods is that the latter has a significantly greater
347 computational overhead, with the need to compare every sequence to every other sequence in
348 its most naïve form.

349 Even within clustering tools, the choice of parameters has been shown to have a critical
350 impact on the results. Whilst a threshold of 97% has become standard, Patin *et al* have
351 shown that 16S rRNA gene sequences as similar as 99% can represent functionally distinct
352 microorganisms, which means that functionally diverse species would be clustered at the
353 97% threshold (86). However, that may rely on accurate sequences, and if those don't exist,
354 the 97% threshold can help avoid over-estimation of biodiversity (87). Susceptibility to
355 differing parameters may also be pipeline-dependent (88). Given the controversy and
356 potential biases of clustering sequences, some have suggested methods and models for using
357 individual sequences to represent OTUs (i.e. remove the clustering step entirely) (89–92).

358

359 **Correcting for gene copy number**

360 Different bacterial species also have varying copy numbers of the 16S rRNA gene (93, 94)
361 which can lead to misinterpretations when comparing the abundance of bacterial OTUs or

362 attempting to construct a "true" description of the microbial community within a sample (95).
363 It is unusual in 16S rRNA gene studies to accurately know the copy numbers for all identified
364 OTUs. Therefore, tools have been developed which seek to correct for copy number
365 variation using sequence databases and phylogenetic information to give a more accurate
366 picture of the relative abundances of these OTUs. These include Copyrighter (96), rrNDB
367 (93), functions in the picante R package and pplacer (97) and part of the PICRUSt package
368 (98).

369 As these techniques are reliant on databases the same problems are present as for taxonomic
370 identification. Principally, lesser studied bacterial taxonomies are less likely to be
371 represented. It is also important to note that when comparing OTUs between samples rather
372 than within a sample (e.g. when comparing treatment effects), the impact of copy number
373 variation is reduced as the under or over representation of OTUs would be consistent across
374 samples as long as the same methodology had been used.

375

376 **Contamination issues**

377 Microbial DNA contamination arising from DNA extraction kits, PCR reagents and the lab
378 environment may have a particularly large effect when studying low microbial biomass
379 samples. Salter *et al* found that contamination in DNA extraction kits not only varied by
380 manufacturer but by individual lot and that samples processed in separate laboratories
381 contained different types of contaminating DNA (99). This lack of predictability led the
382 authors to suggest that "negative" (or reagent-only) controls should be run alongside samples
383 in all 16S rRNA gene metabarcoding studies. If reagent-only controls are not included, this
384 can lead to the misinterpretation of results. When Salter *et al* analysed a dataset comparing
385 nasopharyngeal microbiota samples from children at two time-points they found that while
386 the time-points appeared to cluster separately, this effect was mainly due to bias caused by

387 contamination from the extraction kits used. Randomisation of samples prior to processing
388 may help avoid the introduction of this type of bias. Contamination could also lead to the
389 false identification of microbial communities where they do not in fact exist (100) and could
390 affect our understanding of which bacteria are relevant in clinical samples (101).

391 The amplification of background contaminants from PCR reagents could perhaps be avoided
392 via the use of primer-extension PCR (102) but this would have no effect on contamination
393 originating from other sources. Several methods have been suggested to remove
394 contaminating DNA from reagents and the lab environment including: UV and γ radiation
395 (103–107); DNA intercalation by 8-methoxypsoralen, ethidium monoazide and propidium
396 monoazide (104, 106–108); enzymatic treatments (105–107, 109–111) silica-based
397 membrane filtration (112); $CsCl_2$ density gradient centrifugation (111) and bleach/CoPA
398 solution treatment (105). These methods have shown variable effects on contamination
399 levels and PCR sensitivity and the inclusion of reagent-only controls alongside these
400 decontamination measures is still recommended.

401 What should be done with sequencing data from reagent-only controls is still under debate. It
402 is often not appropriate to simply remove all of the bacterial OTUs found in controls as these
403 may overlap with OTUs which can genuinely be found in samples (108). Other methods
404 have been suggested which take into account the abundance of OTUs to predict the likelihood
405 of sequence reads having originated from contamination. These include an adaptation of the
406 neutral community model (12) and combining qPCR data with OTU relative abundance data
407 to compare the absolute abundance of contaminating OTUs in controls and samples (113).
408 However, the field is rapidly reaching consensus that, due to contamination issues, not
409 including reagent-only controls can negatively impact the quality control of sequence data.

410 **When planning a 16S study, the inclusion of reagent-only controls (i.e. DNA extraction**
411 **kit and PCR controls) is advised.**

412

413 **Conclusions**

414 The study of complex microbial communities using high-throughput sequencing platforms
415 has allowed better understanding of a variety of biological systems and the impact of various
416 conditions (e.g. disease states) on the host microbiome. When considering the literature, it is
417 clear that bias can be introduced into microbiota studies at all methodological stages, from
418 sampling to bioinformatic analysis. While the variety of different 16S rRNA gene
419 metabarcoding methodologies might seem overwhelming, the main factor to keep in mind
420 when designing a microbiota study is consistency. It is paramount to use consistent
421 methodology throughout a study to minimise potential biases which could lead to spurious
422 results.

423 The volume of studies attempting to define best practice for various stages of the microbiome
424 experimental process is large, and we cover only some of the literature in this review.
425 Unfortunately, as can be seen, there is little consensus, and further studies are unlikely to find
426 any. The reality is that many of the biases described in this review are context- and
427 environment- specific, and whilst individual studies may be true within their context, their
428 conclusions may not be transferable to other studies. Clearly, with biases possible at every
429 step, a good experimental design is essential. Recording and publication of all experimental
430 metadata is essential for understanding microbiome studies, and unfortunately many currently
431 published studies lack these data.

432 Trying to find consensus in the literature is challenging, with many studies producing
433 conflicting evidence about the effects of various steps in the experimental process. It is
434 therefore essential that consistency is maintained within a study, and there must be an
435 acceptance that comparison between studies may not be possible.

436 In summary, we recommend extracting DNA from fresh samples if possible; if not, samples
437 should be stored in a consistent manner (i.e. at the same temperature, for the same duration
438 and with or without cryprotectant) with appropriate metadata being recorded. The use of a
439 mechanical lysis step is recommended to minimise potential biases due to some microbial
440 cells being more resistant to lysis. The selection of appropriate primers should be made after
441 careful consideration of the literature, but it is important to note that even universal primers
442 will not amplify all bacteria in a given sample. Sequencing both mock bacterial communities
443 and "negative"/reagent-only controls is important for determining background contamination
444 and sequencing error rate, and should at least be included for each sequencing run and even
445 better, for every batch of commercial reagents/kits. To reduce the chance of OTU inflation
446 caused by sequencing errors, consider complete overlap of MiSeq reads, which translates as
447 targeting a single hypervariable region. Finally, and to re-iterate – record every aspect of
448 your experiment and report it in the methods section and remember that the critical
449 consideration is consistency in methodology at each stage.

450

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459

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