Treating Bugs as Features: A compositional guide to the statistical analysis of the microbiome-gut-brain axis

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Abstract

There has been a growing acknowledgement of the involvement of the gut microbiome - the collection of microbes that reside in our gut - in regulating our mood and behaviour. This phenomenon is referred to as the microbiota-gut-brain axis. While our techniques to measure the presence and abundance of these microbes has been steadily improving, the analysis of microbiome data is non-trivial.

Here, we present a guidebook for the analysis and interpretation of microbiome experiments with a focus on microbiome-gut-brain axis experiments. We give an overview of the types of approaches of microbiome analysis, including caveats and considerations. We emphasize the compositional data analysis (CoDA) paradigm.

Further, this guidebook features an extensive and heavily annotated microbiome analysis in R in the supplementary materials, including a demonstration of volatility analysis and functional gut-metabolic and gut-brain module analysis as a resource for new and experienced bioinformaticians alike.
1 Introduction

Microorganisms can be found in large numbers in almost all environments, including in and on the human body. The largest collection of microbes on humans can be found in the gut and is referred to as the gut microbiome. According to recent estimates, the human gut microbiome typically consists of around $3 \times 10^{13}$ microbes, weighing approximately 200 grams Sender et al. (2016). In terms of genetic diversity, the microbiome outmatches its human host by about two orders of magnitude, and has co-evolved with their eukaryotic hosts Ursell et al. (2012). With the advent of high-throughput sequencing, the gut microbiome has become a popular subject of investigation, as evidenced by large scientific endeavours designed to map the human microbiome to health and disease Claesson et al. (2012); Consortium (2012); Integrative et al. (2019); Peterson et al. (2009); Tigchelaar et al. (2015). As part of these efforts, it has become increasingly clear that the microbiome is in constant bidirectional communication with the host, and that both systems influence each other on multiple levels. For instance, the human gut microbiome has been shown to differ between individuals on the basis of dietary factors, physical health, age, medication, and even psychological health Cryan et al. (2019); Vujkovic-Cvijin et al. (2020). The bidirectional communication between the microbiome and the host brain is referred to as the microbiota-gut-brain axis. There are several ways in which this communication occurs. Prime among these are the production of metabolites like short-chain fatty acids and neuroactive compounds in the microbiome, modulating the immune system and direct stimulation of the vagus nerve Cryan et al. (2019). Besides playing an important role in gut-brain communication during health and homeostasis, the microbiome has also been found to be affected by psychotropic medication Maier et al. (2018); Tomizawa et al. (2020). In some cases, the microbiome can even metabolize psychotropic medication such as L-DOPA, which is commonly prescribed for Parkinson’s disease, altering the concentration of L-DOPA reaching the patient and thus efficiency of the treatment Maini Rekdal et al. (2019). Microbiome analysis involves techniques and theory from a wide array of fields, including molecular biology, genetics, ecology and even mathematical geology. In the case of fields assessing host-microbe interactions, such as the microbiome-gut-brain axis field, expertise from additional fields such as immunology, psychology, psychiatry, pharmacology, neuroscience and nutrition becomes an additional requirement. Similarly, analysing and interpreting microbiome experiments can be challenging, in part because of the sheer number of specialist software tools needed to pre-process and analyse the associated data. This article aims to make microbiome data analysis less daunting by presenting a concise description of the key steps involved. Although there are many reasonable approaches to analysing the microbiome, we set out to provide the reader with at least one such approach. In this article, we present an overview of the various methods used to analyse, interpret, and visualise microbiome studies. While the text below focuses on high-level concepts, we also include a fully reproducible analysis in the Supplement, written in RMarkdown, that takes our readers through a complete start-to-finish analysis of microbiome data. Throughout this article, we use boxes to discuss selected aspects of microbiome analysis more deeply. Ideally, the researchers performing the statistical analysis should be involved during the design stage of the experiment. In practice, this is not always the case. While this article was written with the intent to be helpful at all points of the experimental process, we have given special consideration to the scenario of a bioinformatician coming in after the wetlab experiments have already been completed and the raw data have been gathered. This article and the accompanying supplementary materials were written with an audience specialised in biological psychiatry in mind and many of the examples in this article reflect this. However, we argue the points discussed here can be applied to most, if not all, host-microbiome experiments and indeed microbiome experiments independent of host. An overview of a typical microbiome analysis can be found in Figure 1.
Figure 1: From Stool to Story. Overview of what a typical gut microbiome analysis may look like. In A) Shows the pre-digital part of the pipeline. Genetic material is isolated and digitized, either using 16S or shotgun sequencing. In B) the digitized reads are annotated based on taxonomy and/or function. In C), the features are tallied up into count tables. In D), higher-order patterns within the data, called mesoscale features, are identified. In E) the features of the microbiome are assessed statistically. Finally, in F), the features are interpreted and presented for peer-review.
2 Getting ready for the analysis

2.1 Pre-registration

There is a growing call for the pre-registration of studies. Pre-registration is a mainstay of reproducible science, and is becoming an increasingly common practice Kupferschmidt (2018); Munafò et al. (2017); Nosek et al. (2018). Pre-registration involves documenting hypotheses and an analysis plan for a study prior to examining data and running the analysis. It is a practical commitment to avoid ‘fishing’ and the selective presentation of results on the basis of significance, and to mitigate against the known cognitive biases of human reasoning (e.g., confirmation bias) Wagenmakers et al. (2012). In microbiome science, where the control of the Type 1 error rate is critical, and the reproducibility of findings is particularly challenging Schloss (2018), pre-registration is especially important. Early indications suggest that the practice has reduced publication bias for positive results Allen and Mehler (2019), and can therefore improve the integrity of published research. Pre-registration tools prompt researchers to describe their study and research questions, and then generate a date-stamped document that can be published with a digital object identifier (DOI) either immediately or after a user-defined period of embargo (e.g., following publication). The pre-registration document thus serves as a public record of the planned analyses and analytic strategy which can be referenced in resulting publications to affirm that the findings reflect a hypothesis-driven analysis. Pre-processing steps should also be specified a priori where practicable, since these will affect downstream results. There are a number of free tools and guidelines for pre-registration, including the Open Science Framework (osf.io) and As Predicted (aspredicted.org). These are akin to clinical trial registration sites, and are suitable for observational and experimental studies alike. Guidance as to relevant details to include in pre-registration and study design more broadly can be sought from emerging consensus checklists such as STORMS Mirzayi et al. (2021). It is important to note that within a pre-registration framework, exploratory and post-hoc analyses are still entirely valid. Indeed, within a relatively young and rapidly evolving field such as microbiome science, it is essential to continue hypothesis generation and exploration. However, exploratory analyses should be presented as such, and should be clearly distinguished from confirmatory hypothesis testing Wagenmakers et al. (2012). Pre-registration may include a discussion about the power calculations used to select the sample size, which we discuss in Box 1. The special case of experimental designs involving Faecal Microbiota transplantation (FMT) is discussed separately in Box 5.
BOX 1: Power calculations

Power calculations can be daunting but are an important part of figuring out how many samples you need per group in order to register an effect of a given magnitude as significant. Using power calculations can help avoid two undesirable scenarios: First, it saves us from going through the trouble of running an experiment that would not be able to find any effects even if they actually took place (i.e., an underpowered setup). Second, it saves us from collecting more samples than necessary to test a hypothesis (i.e., an overpowered setup). This second scenario is especially important in the context of animal studies as part of our ethical commitment to avoid excess animal suffering (i.e., “to Reduce” as per the 3Rs of Animal Welfare). Both underpowered and overpowered studies waste precious resources.

In the case of differential abundance analysis, microbiome power calculations differ from other power calculations for one big reason: microbiome data is very high dimensional. Often, the number of features is larger than the sample size. This necessitates an adjustment for multiple testing (e.g., by Benjamini-Hochberg (BH) procedure or Storey’s q-value) which must be accounted for as part of the power calculation. For some adjustments, notably BH, the adjustment depends on the distribution of p-values, which itself depends on the number of true positives. Thus, to account for adjustment, one must estimate how many features will be differentially abundant between treatment groups. It can be hard to estimate this, making it hard to choose the effective FDR-adjusted alpha threshold for the power calculations.

So how to overcome this challenge? At one extreme, we could consider what sample size is needed to detect a single differentially abundant feature if all other features are unaltered. Let’s say only one microbe out of one hundred tested features is actually different. Then, the adjusted p-value needed to reject the null-hypothesis would be one hundred times lower. Thus, to get a conservative sample size, one should perform a power calculation where the significance threshold alpha is divided by the number of features expected to be tested.

At the other extreme, we could consider what sample size is needed to detect a single differentially abundant feature if all features differ between groups. In the case that all individual p-values are lower than the alpha threshold, the BH correction will not adjust any of the p-values to the point that they no longer are under alpha. Thus, to get a liberal sample size, one should perform a power calculation where the significant threshold alpha is not adjusted. From this, we can formulate bounds: For D features, the adjusted alpha used for power calculations should fall between alpha/D and alpha, depending on the number of features we expect to be differentially abundant. For BH adjustment, we recommend a heuristic: adjusted alpha = alpha * M / D where D is the total number of features tested, and M is the total number of features expected to differ significantly between the groups. This adjusted alpha can then be used for power calculations as done under ordinary circumstances.

Once a study is appropriately powered for differential abundance analysis, it seems reasonable to assume that, as a rule-of-thumb, the study is also appropriately powered for testing differences in alpha and beta diversity. Though, to be safe, we recommend that analysts add the total number of planned alpha and beta diversity analyses to D in the formula above.

2.2 16S/amplicon vs. shotgun sequencing

Generally speaking, two methods of microbiome sequencing are widely used:

- 16S sequencing, also called amplicon sequencing, includes methods where an evolutionarily preserved genomic sequence is targeted and sequenced, and
- whole genome shotgun sequencing, where all genetic material in a sample is targeted and sequenced.

While downstream bioinformatics analysis of both types of microbiome sequencing techniques converges, the actual techniques are distinct Clooney et al. (2016). Notably, both techniques are biased towards detecting specific genetic sequences and thus by extent specific microbial taxa Santiago-Rodriguez et al. (2020). Such biases are known to occur between metagenomic sequencing experiments of the same type, even between runs in the same laboratory McLaren et al. (2019).
2.2.1 Pre-processing 16S sequencing data
The analysis of 16S sequencing typically begins by trimming reads, filtering them for quality based on a threshold, and removing chimera sequences. Then, a table of either operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) is generated. The philosophy and process behind these two units differ meaningfully, and this has been comprehensively written about elsewhere Callahan et al. (2017, 2016a). For the purpose of this review, both OTUs and ASVs can be seen as the highest taxonomic resolution that a specific method can distinguish. Roughly speaking, they can be viewed as analogues to species or genera. Although OTUs and ASVs are technically distinct, the two are interchangeable concepts when it comes to downstream statistical analysis. For recommendations on pre-processing 16S data we refer to this excellent workflow Callahan et al. (2016b). After generating a table of OTUs or ASVs, the next step is to assign taxonomy. In most cases, this is done by use of a reference database. Several such databases exist and some are better curated than others. At time of writing, the SILVA database is widely regarded as the most accurate and extensive Balvočiūtė and Huson (2017); Quast et al. (2013). Although the Greengenes database is still often used, it has not been updated since 2013 McDonald et al. (2012). While not covered in this review, the free and open source QIIME2 platform is an excellent and well-maintained Python-based resource for microbiome analysis Estaki et al. (2020).

2.2.2 Pre-processing shotgun sequencing data
In the case of shotgun sequencing, it is also common to filter and trim reads in a fashion analogous to 16S data. Apart from this, non-microbial genetic material needs to be filtered out. This is often done by removing all reads that map to a reference genome of the host organism, as well as any other genomes that may be contaminants (e.g., plant genetic material from diet). Common tools used to process ‘raw’ data from shotgun sequencing include the bioBakery suite McIver et al. (2017), Kraken2 Wood et al. (2019) and Bracken Lu et al. (2017). Typically, taxonomic and functional classification of shotgun metagenomics data is more computationally expensive than its 16S sequencing analysis counterpart and thus often requires a server or computer cluster Simon et al. (2019).

2.2.3 The count table
Although 16S/amplicon and shotgun sequencing differ widely in execution, the type of data that is obtained tends to converge downstream in the analysis. After pre-processing, both 16S and shotgun sequencing methodologies yield a count table. A count table shows how many observations (i.e., counts) there were for each feature (e.g., microbe, function, gene, etc.) per sample. By convention, a count table should have features as columns and samples as rows. Although many software tools assume this organisation, there are notable exceptions, so it is always worth checking the software before proceeding with an analysis. It is tempting to directly correspond a count to a biological instance of a feature in a sample, but due to biases inherent to metagenomic sequencing Dillies et al. (2013); McLaren et al. (2019), raw counts should be pre-processed first, for example via normalization or log-ratio transformation. Sometimes counts are instead expressed as compositional data, which we discuss in Box 2.
In the past few years, awareness is growing that microbiome datasets are compositional, which, if ignored, can lead to spurious results. There are excellent reviews on Compositional Data Analysis (CoDA) in general and how it relates to the microbiome in particular that we encourage our audience to read Calle (2019); Gloor et al. (2017); Quinn et al. (2018). Compositional data analysis theory and practice is continually evolving, and a full review of the field is beyond the scope of this article. In this guidebook, we will recommend, at a minimum, performing a centered log-ratio (CLR) or similar transformation (e.g., PhiLIR) on the count data before performing statistical analysis or visualizing the data. However, there are 3 notable exceptions: First, alpha diversity should not be done on transformed data. Second, stacked bar plots should be generated using counts normalized to 1 or to percentages. Third, correlating taxa to each other, for example as part of a network analysis, warrants special attention. This is because one of the properties of compositional data is that features are inherently negatively correlated. Indeed, Karl Pearson warned against applying his namesake Pearson’s correlation coefficient on compositional data Pearson (1897). Alternatives are available from the propr library Quinn et al. (2017), the SparCC library Friedman and Alm (2012), and the SPIEC-EASI library Kurtz et al. (2015). Note that because microbiome count data typically has many zeros and the logarithm of a zero is undefined, the zeroes in microbiome count data must be addressed. Several reasonable solutions have been proposed, but it remains an open question as to which among these solutions performs best (though c.f., Lubbe Lubbe et al. (2021) for one benchmark). In the demonstration found in the supplementary files, we employ an approach derived from the ALDEx2 framework Fernandes et al. (2014), which samples non-zero values from a Dirichlet distribution. After the CLR-transformation, the values of features can take on any value (unlike count data, which cannot be negative). After transformation, classical statistical approaches can be applied as normal.

2.2.4 Rare features and Rarefaction

Before the microbiome analysis starts, it is common to filter out features by removing them entirely from the count table. Testing fewer features reduces the magnitude of the FDR adjustment penalty, which in turn helps to increase the statistical power for the remaining tests. Most often, the filtered features represent rare taxa or rare genes. Commonly, features that are only detected in a certain percentage of samples are removed. This is referred to as prevalence filtering. Similarly, features that are only detected in low levels can be dropped. This is referred to as abundance filtering. Sometimes, one might wish to filter out features based on other metrics, such as variance Guyon and Elisseeff (2003); Guyon et al. (2002). However, features should not be filtered based on their association with an outcome, as this could bias the p-value estimates in downstream statistical tests. The total number of observations recorded for each sample in a count table depends on the sequencing depth of the assay. Rarefaction is the practice of randomly removing observations from a sample until all samples have the same amount of observations. However, it has been described as an unnecessary and potentially counterproductive measure McMurdie and Holmes (2014). It is more conventional now to address inter-sample differences in sequencing depth through effective library size normalization or log-ratio transformation Aitchison et al. (2000); Quinn et al. (2018). One notable exception is diversity analysis as discussed below Hsieh and Chao (2017); McKnight et al. (2019); Willis (2019). We argue that, while rarefaction is sometimes justifiable and even recommended, rarefaction should not be seen as the default approach.

2.2.5 Dealing with potential confounding

A key challenge of microbiome research, and in particular observational studies of the human microbiome, is delineating the variable-of-interest from other factors that influence the ecosystem. Genetics, ethnicity, early life factors such as modes of birth and feeding and stress, habitual diet, environmental exposures and medication use are just some of the important contributors to the human microbiome, and are also often related to the outcome of interest Dong and Gupta (2019); Maier et al. (2018); Wilson et al. (2020); Yap et al. (2021). These are always worth considering in microbiome research, especially in the context of causal inference modelling. Although not
always the stated aim, causal inference is commonly the underlying motivation for studies of the microbiome. Consider psychiatry, for example. We may aim to estimate the effect(s) that the gut microbiome has on some parameter of brain function, whether it be mood, behavior, cognition, or a neurodevelopmental indicator. Other valid study aims might include description (e.g. of the microbiome in people experiencing depression) and prediction (e.g. can we predict who will develop depression based on their microbial features); however, causal interpretations are often attributed even to these kinds of studies Hernán et al. (2019). Further, even within descriptive or predictive studies, it can be useful to examine if causal features such as dose response or temporality exist. Causal questions are commonly implied even in cross-sectional and associative human studies for example, in which the microbiome is not being manipulated, and its effect is therefore not being explicitly measured. For this reason, causal inference principles have broad relevance. Importantly, causal inference is not the same as assigning causality based on an observational study; rather, causal inference seeks to determine whether the data supports a causal hypothesis by performing statistical analyses within a causal framework. Box 3 summarises the 5 phases of causal inference analysis adapted from Ponsonby, 2021 Ponsonby (2021). These phases make use of a directed acyclic graph (DAG), as shown in Figure 2, modelled on the example from the Zhu et al (2020) dataset expanded on in the accompanying Rmarkdown script.

Figure 2: An example DAG. A DAG describes a hypothetical causal pathway, where an arrow from A → B suggests a causal relationship between A and B. Bidirectional relationships aren’t readily captured within this framework; the dominant direction needs to be selected. This DAG was created using the dagitty R library and reflects variables relevant to the Zhu et al. (2020) schizophrenia dataset used in the accompanying Rmarkdown script. One may wish to include measured variables only, but ideally the framework of constructs and variables is considered prior to (thereby informing) data collection. Some examples of relevant unobserved variables are shown here in grey. For readability, aim to have variables presented in temporal order from left to right. Under the assumptions of this DAG and with only three available covariates (sex, smoking status and body mass index), sex and smoking status comprise the minimal adjustment set required to estimate the total effect of gut microbiota on schizophrenia.
BOX 3: Phases of development for causal inference analysis
Ponsonby (2021) suggests the following phases when developing a causal inference analysis:

A Asking specific, detailed research questions. Build a directed acyclic graph
B Testing the exposure-outcome association. Run unadjusted association analyses
C Consider other variables. Using the DAG, identify potentially confounding variables
D Build multivariable models.
E Evaluate non-causal and causal explanations.

In phase A, a directed acyclic graph VanderWeele and Robins (2007) may be used to identify the hypothesised relationships between the exposure (microbiome), outcome (e.g. schizophrenia) and potentially confounding variables of interest on the basis of prior knowledge VanderWeele et al. (2008). See Figure 2 for an example DAG. Phase B is analysis of the unadjusted associations between exposure and outcome. This will require operational definition of each. For example, which microbial diversity metric will be used? How is schizophrenia assessed? Is the exposure-disease association linear or non-linear in form? In phase C, consider other variables including those with potential causal and non-causal confounding. This could include any common causes of exposure and outcome (e.g. cigarette smoking which may affect both the gut microbiome and risk of schizophrenia), and include any proxy measures of unmeasured common causes of both exposure and outcome (e.g. family history of schizophrenia (if measured) as a proxy for unmeasured genetic factors which could impact both the gut microbiome and risk of schizophrenia) VanderWeele (2019). In addition, consider technical or processing variables that might affect measurement precision e.g. microbiome sequencing batch effects McLaren et al. (2019); Wang and LêCao (2020). It is useful to understand how each of these causal and non-causal potentially confounding variables associate with the exposure and outcome, by examining the associations in the dataset. This will provide information to help assess if the putative factor is a mediator, an antecedent, instrumental variable (antecedent of exposure), or a disease consequence. If so, the putative factor is not a confounder and should not be adjusted for. As well as adjusting for confounding, one may include disease determinants that are independent of outcome Schisterman et al. (2009). Too many variables will challenge the power of small sample sizes, so is dimension reduction a possibility? Is there collinearity or redundancy? The next step, phase D, is then to build multivariable models. Consider refining the a priori DAG on the basis of the data, adding or removing variables as required, and reporting models that are adjusted on the basis of the original DAG, updated DAG and with any additional processing and precision-enhancing variables that reduce measurement error. Whilst it is desirable to investigate all associations manually, DAGitty software Textor et al. (2016) does provide identification of ‘minimal adjustment sets’ which can be used to block all non-causal paths, to estimate the total or direct effects between exposure and outcome. Non-causal confounders such as batch effects do not fit strictly within this tool of causal inference, but comprise an unwanted source of variance that should nonetheless feature in adjusted models Bross (1966). Finally, in phase E, interpret the findings of both unadjusted and adjusted models with due considerations of possible bias, such as measurement and selection bias, and other explanations of effects such as reverse causality.

3 Linking the microbiome to host features

3.1 Diversity indices

The microbiome is a complex ecosystem. The analysis and visualisation of the microbiome can be qualitatively distinct from other high-throughput sequencing data. Although the data arise from a molecular biology assay, several of the statistical approaches used in microbiome analysis originate from other fields, such as ecology. This makes microbiome science a clear beneficiary of interdisciplinary research. Diversity, as popularized in ecology, is a way to quantify and understand...
variation in microbiome samples. Classically, diversity is separated into three related types: Alpha, Beta, and Gamma diversity Sepkoski (1988). Alpha diversity refers to the degree of variation within a sample. Beta diversity refers to the degree of variation between samples. Gamma diversity refers to the total diversity in all samples (which can be thought of as the Alpha diversity of all samples combined). In practice, Gamma diversity is rarely used.

### 3.1.1 Alpha diversity – the diversity within samples

There are many measures of alpha diversity, which can make alpha diversity confusing to understand. It is helpful to classify alpha diversity measures along two axes: the Hill number (0, 1, or 2) and whether it is phylogenetic (yes or no). Regarding the first axis, alpha diversity measures can be understood as being the result of a unifying equation in which a single parameter - called the Hill number - acts to vary the meaning of the equation, and thus define the alpha diversity measure. Every number gives a different alpha diversity metric. In practice, three Hill numbers are most often used: 0, 1 and 2. The number 0 defines Richness, or how many different features a sample has. The number 1 defines Evenness, or how equally the features in a sample are represented (equivalent to Shannon entropy). The number 2 defines Simpson’s Index, or the probability that two features picked at random do not have the same name (as a probability, it is bounded by 0 and 1). Regarding the second axis, other phylogenetic diversity (PD) measures, like Faith’s PD, extend alpha diversity by taking into account the coverage of all features (e.g., bacteria) on a phylogenetic tree. Typically, the more of the tree that is represented in a sample, the higher the diversity. Figure 3 illustrates a classification of several popular alpha diversity measures.

![Figure 3: Alpha Diversity metrics are related to each other. Commonly used alpha diversity metrics in the microbiome field can be classified along two axes. Here, we show the Hill number on the x-axis, and whether the index considers phylogeny on the y-axis.](image)

### 3.1.2 Statistical considerations with Alpha diversity

Alpha diversity is used to summarize the entire microbiome composition as a single number. It is common to model alpha diversity as a dependent variable, using sample meta-data as the predictors. When this is done, the literature has shown that a lower Alpha diversity is often associated with worse host health outcomes in humans Ma et al. (2019). However, it should by no means be
taken as principle that a higher Alpha diversity is strictly “better”, as there are many examples
where elevated Alpha diversity indicates an abnormal or even unhealthy host state. For instance,
in infants there is a high selection pressure on certain microbes, such as numerous species in the
genera Bifidobacterium and Lactobacillus Yang et al. (2019). Here, an increased Alpha diversity
could indicate a lowered selection pressure, which could be indicative of health issues Hill et al.
(2017). There are at least three issues to consider when using alpha diversity for microbiome data
analysis.

- First, all alpha diversity measures are sensitive to transcript-level measurement biases such as
  PCR bias McLaren et al. (2019). This is recognized as a critical limitation of alpha diversity
  that cannot be resolved unless the PCR bias factors are already known a priori McLaren
  et al. (2019).

- Second, some alpha diversity measures will change depending on the total number of observa-
tions (i.e., feature counts) recorded for a sample. It is often appropriate to “normalize” away
differences in sequencing depth before comparing alpha diversity between samples. This can
be done by dividing out total counts to get proportions (e.g., in the case of Shannon entropy),
or by performing rarefaction. Both procedures will equalise the number of observations be-
tween samples, so that they can be compared more fairly. In fact, many alpha diversity
software tools will perform this “normalization” step automatically, though no normalization
step is perfect.

- Third, all alpha diversity measures are sensitive to the number of rare taxa that get observed
in samples, and thus are sensitive to sequencing depth. Failure to record the presence of a
rare taxa, when it is in fact present, can make a sample appear less diverse than it is Willis
(2019).

It is important to keep these three issues in mind when interpreting the results from an alpha
diversity analysis. For example, in the case of very low microbial load due to, say, an antibiotics
course, alpha diversity may appear higher than expected Elokil et al. (2020). This seemingly
paradoxical phenomenon can be better understood when considering that there is a limited amount
of sequencing material during the sequencing process, regardless of method used. In the case of
an abundance of microbes, the most prevalent ones will use up most of the sequencing reagents,
leaving little for the rarer taxa to be sequenced. In the case of a low bacterial load, there are no
prevalent microbes to take up most of the material and thus the rarer taxa that happen to be
in the sample will have a much higher likelihood to be sequenced, thus inflating the calculated
diversity.

3.1.3 Beta diversity – the diversity between samples

Beta diversity refers to the degree of difference between two microbiomes. It is worth appreciating
the assumptions and limitations that come with describing the total difference between two complex
ecosystems as a single number. There are many ways to measure the “difference” between two
samples, and each one imparts a unique perspective on the data. In principle, one could use any
dissimilarity or distance measure. Three common difference measures are:

- Jaccard’s Index: This is a similarity measure that simply describes the proportion of unique
taxa that are shared between two samples, without taking abundance into account. As such,
one could interpret Jaccard’s Index as the fraction of unique taxa (not abundances) shared
by two samples. If two samples have exactly the same microbe taxa, the Jaccard index will
be 1. In the case that two samples share no microbe taxa, the Jaccard index will be 0.
Subtracting Jaccard’s index from 1 makes it the Jaccard Distance measure.

- Euclidean Distance: This is the geometric distance derived by applying the Pythagorean the-
orem, using every microbe as a separate dimension. It is computed by taking (the square root
of) the sum of the squared differences in bacteria abundance. As in geometry, the minimum
Euclidean distance is 0 while the maximum is unbounded. Euclidean Distance satisfies the
triangle inequality, making it useful for certain geometric analyses, such as volatility analysis
as discussed below. A related measure called Aitchison Distance is the Euclidean distance
between log-ratio transformed data. This distance has a favorable property known as sub-
compositional dominance (i.e., the removal of a taxa feature will never make two samples
appear further apart) and is also equivalent to taking the Euclidean distance between all pairwise log-ratios Aitchison et al. (2000).

- Bray-Curtis Dissimilarity: This dissimilarity measure is similar to Jaccard’s Index in that it ranges from 0-to-1, while also being similar to Euclidean distance in that it is computed from the differences between abundances. Bray-Curtis is calculated by summing the difference in abundance between each microbial taxon, and dividing it by the total microbial abundance of the two samples. Thus, one could interpret Bray-Curtis as the fraction of abundances unshared by two samples (compare with Jaccard Distance, which is the fraction of unique taxa unshared by two samples).

The three common difference measures listed above make use of bacteria presence or abundance without considering the phylogenetic relationship between the bacteria. Just as we can make alpha diversity phylogenetic, we can do the same with beta diversity.

- UniFrac: This distance makes use of phylogenetic information to measure the difference between samples. There are (at least) two types. The Unweighted UniFrac Distance considers the branch lengths of the phylogenetic tree along with microbial presence, and is defined as the sum of branch lengths unshared between the samples divided by the sum of branch lengths present in either sample. This measure has some analogy to Jaccard Distance in that an unweighted UniFrac distance of 1 means the two samples share no bacteria taxa in common. The Weighted UniFrac Distance further considers microbial abundance, and weighs each branch length in the Unweighted UniFrac formula by per-sample proportional abundances.

- PhILR: This method uses a log-ratio transformation called the isometric log-ratio (ILR) transformation which uses a phylogenetic tree to recast the microbiome variables as a series of log-contrasts called “balances” Silverman et al. (2017). PhILR offers 2 weighting options called taxon weighting and branch weighting. When both are disabled, the PhILR beta diversity is equivalent to Aitchison distance, although its use of phylogeny-based coordinates may yield a more interpretable ordination of the data. The taxon weighting provides a compositionally robust alternative to weighted Jaccard or Bray-Curtis measures, while the branch weighting provides a compositionally robust alternative to UniFrac measures.

Figure 4 illustrates a decision tree that we as the authors use when selecting a beta diversity measure. As with alpha diversity, it is sometimes helpful to compare and contrast the results from multiple measures of beta diversity.

3.1.4 Statistical considerations with Beta diversity

There are two general strategies used to assess beta diversity:

- Qualitative – visualization of samples plotted across an ordination of the data, such as a principal components analysis (PCA) or principal coordinates analysis (PCoA)

- Quantitative – explicit modelling of PCA/PCoA axes as a dependent variable, using the sample meta-data as the predictors, or some other formal comparison between the group centroids

When discussing Beta diversity, it is important to consider that microbiome data are compositional. This is because some common difference measures can have an irregular behavior when applied to compositional data (most notably, Euclidean distances). Fortunately, the study of compositional data has allowed for the development of tools and transformations that enable us to work with compositional data in virtually the same manner as regular data. In the case of Beta diversity, the preferred alternative to Euclidean distance is Aitchison distance Aitchison et al. (2000). One clear advantage of Aitchison distance, which applies to the unweighted PhILR distance too, is that - unlike alpha diversity and other beta diversities - it is unaffected by PCR bias McLaren et al. (2019). We note here that there is an interesting parallel between compositional data and the probability vectors routinely studied in information theory. Similar to how Shannon entropy can be used to measure alpha diversity, other informatic metrics like Kullback-Leibler divergence could feasibly be used to measure beta diversity Erb and Ay (2021). Although these metrics are not commonplace in microbiome analysis, they are often used in machine learning, and may be
Figure 4: Decision tree featuring common Beta diversity indices. Some Beta diversity indices are more suitable depending on the needs of the researcher. This decision tree recommends an index based on three common criteria: whether one wants to consider abundance, phylogeny, and/or true distance.
more robust for the analysis of amalgamated data, for example genus-level or family-level counts Quinn and Erb (2020).

3.1.5 Volatility

The microbiome is a dynamic ecosystem and undergoes constant change. The degree of change in the microbiome over time is called volatility, which is inversely related to stability. It can be helpful to think of volatility as a change in sample diversity (alpha or beta) over time. In a neutral setting, without intervention, a higher volatility is generally considered to be associated with negative health outcomes Bastiaanssen et al. (2021). One way to calculate volatility is to measure the beta diversity between two or more time points corresponding to the same host. When measuring volatility in this fashion, it is especially useful to choose a beta diversity metric that is also a distance (i.e., follows triangle inequality, like PhiLR or Aitchison distance) so that any comparisons are standardized for all time points. Volatility has been recently shown to differ between enterotypes (which are discussed below), indicating that microbiome composition at least partially explains microbiome volatility Vandeputte et al. (2021).

3.2 Differential feature abundance

3.2.1 Taxa and Genes

Differential abundance (DA) analysis is perhaps one of the most popular microbiome analyses. Like alpha and beta diversities, there are many approaches to measuring DA. Most methods follow the same general pattern: (a) apply a normalization to correct for variation in sequencing depth and/or other biases; (b) perform a univariate statistical test for each taxon as a dependent variable with the sample meta-data as predictors; and (c) adjust the p-values for multiple testing, for example using Bonferroni, Storey’s q-value or Benjamini-Hochberg. We expand on multiple testing corrections in Box 4. Note that while most DA software do treat taxa as the dependent variables, one could just as well treat taxa as the predictors, as routinely done in machine learning applications.
**BOX 4: Adjustment for multiple testing**

Often, the amount of features per sample is higher than the sample size. This property of microbiome data necessitates an adjustment accounting for multiple testing (e.g., often by controlling the false discovery rate (FDR) using a method like the Benjamini-Hochberg procedure or Storey’s q-value Storey and Tibshirani (2003)). Multiple testing must be considered as part of the power calculation. This is because without an FDR correction and using a traditional \( p < 0.05 \) threshold, approximately one in twenty tests would give a false positive result, making the \( p < 0.05 \) threshold too lenient of an evidence threshold for most applications. Unfortunately, despite the necessity of FDR in these types of experiments, it can be unclear how to adjust power calculations when FDR is involved. The severity of FDR is dependent on the \( p \)-values that are to be adjusted. The Benjamini-Hochberg procedure, which is the most common FDR method in microbiome research, adjusts every \( p \)-value by multiplying them by the total number of tests to be corrected and then dividing that number by the amount of \( p \)-values that were found to less than or equal to that \( p \)-value to begin with. In that way, if there are many low \( p \)-values in the batch, the FDR correction will be less severe than if there are only few low \( p \)-values to begin with. Unless there is a good reason not to do so, researchers should always correct for multiple testing in microbiome and other high-dimensional datasets.

Although DA analysis is commonplace among microbiome studies, simulations have shown that many popular DA methods do not adequately control the false discovery rate even with FDR adjustment Hawinkel et al. (2019). It is also important to note that the quality of a differential abundance analysis depends in part on the propriety of the normalization used Quinn et al. (2018). As such, it is important to know and assess the assumptions that underlie the normalization procedure. Many normalizations imply an assumption that “the majority of the taxa are unchanged between samples”, an assumption that may not hold for all microbiome studies (for instance, in the case of microbiome depletion using antibiotics or when comparing the microbiomes from completely different body sites). This is also the case when using the centered log-ratio transformation as a normalization for DA analysis Erb and Notredame (2016). In the case that the assumptions of differential abundance analysis do not hold, mesoscale analyses like balance selection and data-driven amalgamation, as discussed below, may provide a more robust alternative. If the reader wishes to perform a DA analysis, they should keep in mind that DA results are often sensitive to the normalization used. Moreover, zero imputation may be required if using a normalization or transformation that involves taking the logarithm, as many do. A benchmark of zero imputation is available from Lubbe et al. (2021), which recommends replacing zeros with a small number drawn from a random uniform distribution. It is often advisable to evaluate how different normalization methods, and zero imputation strategies, change the results.

### 3.2.2 Functions

Another line of investigation that starts at this point is functional inference. There are two general strategies to measuring whether the functions of the measured taxa (or genes) associate with the sample meta-data. Both approaches require some external database that assigns functions to the taxa (or genes), which we will term a functional database. The strategies are:

- **Primary analysis**: In this approach, the functional database is used to score each function based on the taxa or gene abundances. This produces a functional count table, which is simply a count table tallying the occurrence of functions rather than taxa or any other feature. Then, one could proceed with a routine statistical analysis that uses the functional scores as a statistical variable, just as one would do for a microbiial count table. Functional count tables are also a useful strategy for engineering interpretable features for machine learning applications Beykikhoshk et al. (2020). When performing a compositional data analysis, one could convert relative abundances of microbes to relative abundances of pathways by taking the dot product between (a) a table of proportions (i.e., with counts scaled so that each sample, as rows, sums to 1) and (b) a scaled functional database (i.e., with pathway membership scaled so that each feature, as rows, also sums to 1). For \( N \) samples, \( G \) genes, and
P pathways, the dot product between $[\text{NxG}]$ relative abundances and $[\text{GxP}]$ scaled pathway scores will yield a new table of $[\text{NxP}]$ relative abundances.

- Secondary analysis: In this approach, a DA analysis is first performed on the taxa or genes, and then the functional database is used to summarize the DA results. In the simplest case, the DA results can be dichotomized into significant or non-significant, and functional status can be dichotomized as present or absent. For each function, one could perform a Fisher exact test (or similar) to measure whether that function is over-enriched among the significant taxa or genes Chong et al. (2018); Irizarry et al. (2009). Gene set enrichment analysis (GSEA) is a popular generalization of this concept, and is commonplace in gene expression analysis Irizarry et al. (2009).

The way in which one gets to the functional count table depends on the type of sequencing. In the case of 16S, Piphillin and PICRUSt2 are two options. Both of these tools infer what the metagenome of a sample might look like by mapping 16S sequences to a functional database of fully sequenced microbial genomes (e.g., KEGG or MetaCyc), then inferring a functional count table based on the functions present in the reference genomes. In the case of shotgun sequencing, we infer function directly. Since the full microbial sequences are already available, we only need to identify genes and annotate them, for example with the same KEGG or MetaCyc database. Tools like Woltka or HUMAnN3 in the biobakery suite are typically used to generate a functional count table for shotgun data. In comparing 16S with shotgun sequencing, 16S functional inference can be thought of as a bigger inferential leap than shotgun. With 16S, we have to first guess the entire genomic content based on a single sequence before inferring function, rather than inferring function directly from the detected genes (as done in shotgun sequencing). In both cases, a functional analysis is limited by the validity and completeness of the functional database used to assign functional importance to the taxa or genes. The number of functional databases is currently quite limited, with KEGG, UniRef90 and MetaCyc being among the most common. Like taxonomic databases, functional databases are updated frequently and results may be affected as a consequence. In general, the functional microbiome is known to be more consistent between hosts than the taxonomic microbiome, meaning that the results of functional analyses might generalize better Mehta et al. (2018). As databases expand over time, it is important to report the version number as part of the Methods.

3.3 Multivariate approaches

3.3.1 Stratification / Clustering of samples

In some cases, it may be necessary to stratify one’s data into clusters, i.e., to cluster samples into distinct sub-groups based on their microbiome signature. In the case of microbiome data, stratification is commonly done to define enterotypes, a term describing large subgroups based on microbial taxonomic composition in healthy humans. The precise number of true enterotypes, as well as the best way to detect them, is still up for debate (though 3-4 enterotypes are often cited Costea et al. (2018); Vandeputte et al. (2017)). Enterotypes appear to be important constructs because they are related to factors like host health, diet and exercise, though have known limitations Knights et al. (2014). Notably, bacterial load—which is not easy to estimate from 16S and shotgun data (although c.f., Cruz et al. (2021)), but can be assessed by pan-bacterial qPCR or combination FISH and cell-sorting—is associated with enterotype identity and may bias results Costea et al. (2018); Knights et al. (2014); Vandeputte et al. (2017).

While stratifying samples based on feature abundance can be reasonable, for instance when pursuing functional groups of microbes that might exhibit competitive exclusion, it is rarely advisable to stratify samples into subgroups while in the middle of an analysis of data with only 10s-100s of samples because there are too few samples for validation. It is especially important to validate data-driven stratifications, either in a new cohort or in a subsection of withheld data that can be used as a validation set. Spurious strata can frequently arise from technical or biological artifacts, leading enthusiastic researchers on long and fruitless tangents. Clustering algorithms, by design, will cluster, and can even find impressive clusters among random noise!
3.3.2 Multivariate analyses

Researchers are often interested in associations between taxa and other measured host features, such as clinical meta-data or secondary measurements like metabolomic profiles. This is a slightly more difficult task because we must consider two datasets at once, making the problem multivariable in nature. This approach is becoming increasingly common as the microbiome and metabolome are more routinely collected in parallel Lloyd-Price et al. (2019); Smolinska et al. (2018); Tang et al. (2019); Yachida et al. (2019). There are 3 general approaches to data integration:

- **Univariate-univariate**: Even when having access to two separate multivariable data sets, one can still perform an acceptable analysis using “simple” univariate methods by correlating each microbiome feature (e.g., taxa or gene) with individual features in the other data set, one at a time.

- **Univariate-multivariate**: Another good approach involves treating one feature from one data set as a dependent variable, and using all features from the other data set as the predictors. By repeating this for each feature, one at a time, the analyst can describe all associations between the data sets.

- **Multivariate-multivariate**: Finally, one could use multivariate regression, such as a canonical correlation analysis or redundancy analysis Meng et al. (2016), to get a single model that associates all features from one data set with all features from the other data set. The mixOmics package provides a user-friendly implementation of multivariable methods for microbiome research Lê Cao et al. (2008); Rohart et al. (2017). Similarly, neural networks can be used to identify multivariable associations Le et al. (2020); Morton et al. (2019); Reiman et al. (2021).

Whatever approach one uses, analysts should take care to normalize or transform their data appropriately, especially since correlations can yield spurious results when measured for compositional data Quinn et al. (2017); Quinn and Erb (2021). As with differential abundance analysis, analysts who perform multiple multivariate tests should apply an FDR-adjustment to their results. When multivariate methods do not accommodate straightforward null hypothesis testing, analysts may wish to use permutations or algorithmic validation (e.g., cross-validation) instead.

4 Exploring the mesoscale

4.1 Mesoscale features (Guilds, GBMs, networks)

Mesoscale features of the microbiome are features that do not necessarily contain information about its smallest parts (the microscale), nor about the whole system (the macroscale), but rather contain information about patterns within parts of a microbiome that can be seen across samples. Mesoscale analysis focuses on identifying community-level patterns that define the ecosystem(s) under study. The mesoscale is an important object of study in theoretical ecology Hogeweg (2010). Here, some of the more common types of microbiome mesoscale features will be discussed.

4.1.1 Knowledge-based mesoscale features

- **Ecological Guilds**: Ecological guilds are taxonomically unrelated but functionally related clusters of microbes that have a shared role in the microbiome (e.g., occupy a common niche). For example, microbial communities across a wide span of environments including soil, the ocean, and the human gut could be assigned to trophic groups based on how they feed on one set of substrates and subsequently pass on metabolites to another trophic group Gralka et al. (2020). While ecological guilds are a promising concept in microbiome science, to our knowledge there are currently no standardized pipelines or databases that can be used to detect and compare ecological guilds across cohorts and experiments. Such tools would be welcome additions to the field Lam et al. (2018); Zhao et al. (2018).

- **Functional Modules**: Functional modules are a list of curated metabolic pathways encoding for processes that are related to a specific aspect of the microbiome. There exist two classes of functional modules that we will consider here. Gut-Brain modules cover pathways that are related to gut-brain communication, such as serotonin degradation or histamine...
production. The complete list of Gut-Brain Modules can be accessed as a table in the supple-
mentary files of the paper that introduced them Valles-Colomer et al. (2019) as well as online
(http://raeslab.org/software/gbms.html). Gut-Metabolic Modules cover metabolic processes
in the microbiome. Changes in Gut-Metabolic Modules can indicate a shift in the microbial
metabolic environment and thereby in the fitness landscape, thus allowing for microbes with
different metabolic features to thrive. The complete list of Gut-Metabolic Modules can be
accessed as a table in the supplementary files of the paper that introduced them Vieira-Silva
et al. (2016) as well as on GitHub (https://github.com/raeslab/GMMs). Functional modules
are especially useful because they can be very easy to interpret and sometimes help develop
hypotheses for future experiments.

4.2 Data-driven mesoscale features

- **Network analysis:** Network analysis is most often applied to study or visualize associations
  between microbiome features like taxa or genes. This requires some measure of association,
  for which many exist, the most popular being Pearson’s correlation. However, correlations
  have been shown to yield spurious results when applied to compositional data Lovell et al.
  (2015). For this reason, several alternatives have been designed specifically for microbiome
data Friedman and Alm (2012); Kurtz et al. (2015); Quinn et al. (2017), also see box 2 on
compositionality. These metrics build upon a log-ratio transformation which makes them
more robust to the biases introduced by compositionality Quinn et al. (2018), though they
  can still be prone to false positives Erb and Notredame (2016). A recent benchmark of
213 single-cell data sets has shown that proportionality has excellent performance for sparse
high-dimensional data like those encountered in microbiome research Skinnider et al. (2019).

- **Balance selection and summed log-ratios:** Balance selection and data-driven amalga-
mation are two new approaches to learning mesoscale features directly from the data. In
both cases, the motivation is to find mesoscale features that serve as a biomarker to predict
another variable-of-interest. These mesoscale features are unique in that they are defined
explicitly as a ratio between groups of taxa, similar to the Firmicutes-to-Bacteroidetes ratio
previously identified as a useful biomarker Mariat et al. (2009). By using a ratio of taxa, any
normalization factors would cancel, thus making the method normalization-free. When the
groups of taxa are summarized by a geometric mean, the resultant mesoscale feature is called
a balance. When they are summarized by a sum, the resultant mesoscale feature is called a
summed log-ratio (SLR). Software tools like selbal Rivera-Pinto et al. (2018), balance Quinn
(2018), amalgam Quinn and Erb (2020), and CoDaCoRe Gordon-Rodriguez et al. (2021)
enable analysts to learn mesoscale features in a few lines of code. Generally speaking, it is
customary to validate the reliability of these features by measuring predictive performance
in a withheld test set Quinn et al. (2021).
BOX 5: Faecal Microbiota Transplantation (FMT)

Faecal Microbiota Transplantation involves transferring the microbiome from a donor to a recipient host, often after the recipient microbiome has been washed out using antibiotics or by inducing diarrhea. The idea behind the procedure is to transfer a microbial ecosystem, and potentially the influence said ecosystem has on its host along with it. Often in preclinical studies, murine models are used as the recipient of human microbiomes. FMT can be a useful tool to establish that a phenotype can be transferred by the microbiome, implying that the microbiome is a causal factor in the development of a phenotype. FMT experiments have been used in experiments that provide evidence for the microbiome-gut-brain axis, including in depression and aging Boehme et al. (2021); Kelly et al. (2016). While FMT can be a powerful experimental tool, experimental designs involving FMT are non-trivial and have been the subject of valid criticism Walter et al. (2020). One such criticism is that observational and experimental units are often conflated in FMT studies. Observational units refers to the number of recipients, whereas experimental units refers to the number of donors. Essentially, the criticism is that in a study where a behaviour phenotype is transferred from a patient donor to twenty mouse recipients, the N-number is not twenty but rather one, as there was only one donor microbiome that transferred the phenotype. Pooling donor faecal microbiome samples comes with drawbacks and should not be considered the default option, for several reasons. First, pooling masks the inter-donor variance of the microbiome, which makes it difficult to trace back and investigate what features of the donor microbiome may have caused a phenotype to be transferred. Second, pooling produces microbiome compositions never found in nature. It is well known that numerous taxa display competitive exclusion, i.e. they never stably appear in the same ecosystem. Pooling can therefore create unstable microbial ecosystems, which may end up in distinct compositional equilibria in the recipients. The recommendation is therefore to power the study based on the number of donors rather than number of recipients Gheorghe et al. (2021). Currently, inter-recipient variance in microbiome composition is hard to estimate due to the differences in methodology between FMT studies and we recommend taking several recipients per donor to estimate the inter-recipient variance in colonization, which may also be dependent on the donor. In terms of statistics, we suggest generalized linear mixed models to account for inter-donor variance, using donor ID as a random effect. These types of models can for instance be found in the highly cited R library lme4 Bates et al. (2015).

5 Discussion

The design of host-microbiome experiments and the analysis and interpretation of the resulting data can be a daunting task. In this guidebook review, we set out to highlight the most common pitfalls and to provide the reader with the tools to perform their own analysis. We do not claim this approach is the only reasonable way to perform microbiome analysis, only that it is a reasonable one. Generally, host-microbiome studies would benefit from reporting a characterization of the microbiome data in terms of alpha diversity, beta diversity and the general microbial composition using stacked bar plots or similar. During the statistical analysis of microbiome data, including modelling, correlating and differential abundance testing, it is important to consider the compositional nature of microbiome data, for example by first performing a CLR-transformation, and to account for the large number of tests performed, for example by performing the Benjamini-Hochberg procedure. In many cases, studies would also benefit from considering the functional potential of the microbiome rather than limiting analysis to the level of taxonomy. This guidebook review is not without limitations, we acknowledge this guidebook review cannot cover the entire field of microbiome bioinformatics analysis. Indeed, we chose not to cover in silico metabolic modelling of the microbiome. We also focus on metagenomic analysis of the microbiome, rather than other methods to interrogate the microbiome such as metabolomic, metaproteomics or metatranscriptomics. The microbiome field is currently undergoing a phase of rapid growth and development. We anticipate that new tools, databases and approaches will slowly replace the current suite. In particular, we anticipate a movement towards more longitudinal experimental designs, as well as the integration of multiple omics approaches on the same microbiome in order to more clearly capture the metabolic functional capacity of the microbiome. In terms of statistical analysis, we
encourage the ongoing adoption and further development of CoDa-oriented methodology. Open, freely available and well-documented resources like Bioconductor and CRAN are, and will continue to be, essential for the development and adoption of new bioinformatics tools and pipelines in the microbiome field as well as the broader scientific community. Lastly, we would like to highlight that one of the major strengths of science is to build upon the previous findings of others. Often in the microbiome field, a large amount of data is gathered and a broad analysis is performed, perhaps linking the microbiome to a host condition. Whenever possible, raw sequencing data should be made publicly available as it is essential for reliable and robust meta-analyses. While these types of large-scale studies with broad hypotheses remain valuable to map out the interplay between the microbiome and host, we argue the field is ready to move towards hypothesis-driven experiments with the intent to uncover specific mechanisms and even tractable aspects of the microbiome to help improve both our understanding of the microbiome and our general health and wellbeing. These types of specific hypotheses should be formed based on observations from the large-scale exploratory studies and would also benefit from having bioinformaticians and biostatisticians present during the design stage.

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Conducting a Microbiome Analysis

Thomaz F. S. Bastiaanssen

0. Introduction

Here, we will demonstrate how a microbiome analysis may look in practice. For this demonstration, we have adapted some shotgun metagenomic data from the curatedMetagenomicData library in R. We’re looking at a human cohort starring in the Metagenome-wide association of gut microbiome features for schizophrenia study (DOI: 10.1038/s41467-020-15457-9). After downloading the data it was simplified by summing together all strains by genus. This will make it easier to analyse without access to a server. Briefly, in this data set, we have WGS data from faecal samples from both patients with schizophrenia and healthy volunteers, which will be referred to as “healthy” in the Legends. This data has been included in the Tjazi library on github for easy access purposes. Notably, we’ll be using smoking status and sex to demonstrate including a covariate in the analysis. All R code used to transform, wrangle (reorganise) and plot the data is also shown below as to hopefully provide a toolkit for aspiring and veteran bioinformaticians alike. It should be noted that the analysis performed here may not perfectly correspond to the one performed in the original 2020 manuscript, nor does the outcome (though they do generally agree in that there is an effect of schizophrenia in the microbiome). This is expected and indeed very common for microbiome studies. It is the result of using a different statistical paradigm and should in no way discredit the original analysis.

At the end of this document, we have included a few excursions into more advanced subjects we find useful, but that did not necessarily fit in with the mainline analysis.

Code chunk: Load our libraries

```r
#Statistical tools
library(vegan)
#install.packages("vegan")
library(iNEXT)
#install.packages("iNEXT")
library(Tjazi)
#devtools::install_github("thomazbastiaanssen/Tjazi")

#Data Wrangling
library(tidyverse)
#install.packages("tidyverse")
library(knitr)
#install.packages("knitr")
library(waldo)
#install.packages("waldo")

#Plotting
library(ggplot2)
#install.packages("ggplot2")
library(ggforce)
#install.packages("ggforce")
library(patchwork)
#install.packages("patchwork")
library(ggbeeswarm)
#install.packages("ggbeeswarm")
library(metafolio)
#install.packages("metafolio")

#Load prepared data from the schizophrenia study stored in the Tjazi library
data(guidebook_data)
```
Code chunk: Load our count table and perform the CLR-transformation

```r
#Disable strings automatically being read in as factors to avoid unintuitive behaviour.
options(stringsAsFactors = F)

#Set a seed for the purposes of reproducibility in this document.
set.seed(1)

#Load in the genus level count table and the metadata file.
#Since we're using prepared data, we already loaded it using `data(guidebook_data)`,
#but typically we'd do something like this:
#
#counts <- read.delim("genus_level_counts.csv", sep = ",", row.names = 1, header = T)
counts <- counts ; metadata <- metadata
#To be safe, let's check whether our metadata and our count table have the same names.
print(waldo::compare(sort(metadata$master_ID), sort(colnames(counts)), max_diffs = 5))

## old   | new
## [1] "wHAXPI032581-18" - "wHAXPI032581.18" [1]
## [2] "wHAXPI032582-19" - "wHAXPI032582.19" [2]
## [3] "wHAXPI032583-21" - "wHAXPI032583.21" [3]
## [4] "wHAXPI032584-22" - "wHAXPI032584.22" [4]
## [5] "wHAXPI032585-23" - "wHAXPI032585.23" [5]
## ... ... ... and 166 more ...

#Looks like the metadata names contain dashes whereas the count table contains points.
#We'll change the dashes into dots in the metadata file.
metadata$master_ID <- gsub(metadata$master_ID, pattern = "-", replacement = ".")

#Reorder the columns based on the metadata.
counts <- counts[,metadata$master_ID]

#Fork off your count data so that you always have an untouched version handy.
genus <- counts

#make sure our count data is all numbers
genus <- apply(genus,c(1,2),function(x) as.numeric(as.character(x)))

#Remove features with prevalence < 10% in two steps:
#First, determine how often every feature is absent in a sample
n_zeroes <- rowSums(genus == 0)

#Then, remove features that are absent in more than your threshold (90% in this case).
genus <- genus[n_zeroes <= round(ncol(genus) * 0.90),]

#Perform a CLR transformation
genus.exp <- clr_c(genus)
```


Intermezzo: Interpreting Centered Log-Ratio Transformed Data

The centered log-ratio (CLR) transformation may be the most common approach to deal with compositional data, such as microbiome sequencing data. We will not go into the reasons why this transformation is used here - see the main text - but we will provide some material to help form intuition on what the CLR transformation does and how to interpret it. Let’s start by taking a look at the mathematical notation.

Let’s say we have microbiome sample which we will treat as a vector called \( \mathbf{x} \) with size \( D \). We’ll refer to the taxa - or more generally the elements - of this vector \( \mathbf{x} \) as \( x_1 - x_D \). Then, CLR-transforming that vector \( \mathbf{x} \) would look like this:

\[
\text{clr}(\mathbf{x}) = \left\{ \ln \left( \frac{x_1}{G(\mathbf{x})} \right), \ldots, \ln \left( \frac{x_D}{G(\mathbf{x})} \right) \right\}
\]

Where \( G(\mathbf{x}) \) is the geometric mean of \( \mathbf{x} \). Let’s go through it step by step.

You can calculate the geometric mean of a set of \( n \) numbers by multiplying them together and then taking the \( n \)th root. Just like the ‘regular’ mean, the geometric mean says something about the center of your data.

Essentially what this says is that in order to get the CLR-transformed values of a vector, you take every element of that vector, divide it by the geometric mean of the entire vector and then take the natural logarithm of the result and you’re done.

We can deduce a few things about this transformation.

- First, since we’re taking a natural logarithm, \( \frac{x_n}{G(\mathbf{x})} \) can never be zero as the logarithm of zero is undefined. This means that we need to either replace or remove every zero in our data before we use this transformation. We expand on strategies for this in the main text.
- Second, the possible range of our data has changed. Regular counts can go from 0 to infinity and relative abundance data can go from 0 to 1, but CLR-transformed data can go from negative infinity to positive infinity. The logarithm of a very small number divided by a very large number will be very negative.
- Third, if \( x_n \) is exactly the same as the geometric mean \( G(\mathbf{x}) \), \( \frac{x_n}{G(\mathbf{x})} \) will be 1 and thus \( \text{clr}(x_n) \) will be 0 as the logarithm of 1 is equal to 0. This gives us some intuition about the size of CLR-transformed values. Going further on this, it means that an increase of 1 on a CLR-transformed scale corresponds to multiplying with \( e \), Euler’s number, which is approximately equal to 2.718282. Conversely, a decrease of 1 on a CLR-transformed scale corresponds to dividing by \( e \).

Furthermore there are a few points to keep in mind when interpreting CLR-transformed values.

- First, the CLR-transformation is especially useful in the scenario where most features do not change, so that the geometric mean remains reasonably stable between your samples. If the geometric mean is very different between your samples, you’re dividing by very different values between your samples.
- Second, especially for microbiome sequencing experiments, we are usually dealing with how many reads we found for any given organism. Typically, we cannot relate this back to the absolute or even the relative abundances of those organisms, as all microbes have their own microbe-to-reads conversion rate (again see the main text). Even so, the ratios between the reads are still highly informative.

The CLR-transformation is not a perfect solution for compositionality - in fact the idea of a solution to a type of data seems a little odd - but in practice the CLR-transformation tends to be a handy tool on the belt of a bioinformatician. Understanding what exactly it does will greatly improve its utility and reduce the chance of misinterpreting an analysis.
1. Stacked Barplots

Stacked barplots provide a visually appealing overview of the composition of each sample. Normally, no tests are performed here, but they can be helpful to give the data a visual check over and to show obvious shifts in composition between groups. They could be seen as a mix of alpha and beta diversity, as you can look at both the composition of a single sample (alpha) and how much the samples differ from each other (beta).

Code chunk: Generating a stacked barplot from a count table

```r
# Fork off from the untransformed counts table
bargenus <- counts

# Make into relative abundance
bargenus <- apply(bargenus, 2, function(i) i/sum(i))

# Define a cutoff for rare taxa in several steps:
# first, determine the max % abundance every feature ever shows up at
maxabundances <- apply(bargenus, 1, max)

# Meanwhile, transpose the count table for future wrangling.
# For every sample, sum up all rare taxa ( < 1% at their highest in this case)
bargenus$`Rare Taxa` <- rowSums(bargenus[,maxabundances < 0.01], na.rm = TRUE)

# Remove the individual rare taxa now that they're summed up
bargenus = bargenus[,c(maxabundances > 0.01, T)] # `T` to include the `Rare Taxa`

# Prepare the data for ggplot by adding in metadata here
bargenus$Group = metadata$Group
bargenus$ID = metadata$master_ID

# Wrangle the data to long format for easy plotting
barlong = bargenus %>%
  pivot_longer(!c(ID, Group), names_to = c("Microbe"), values_to = "value") %>%
  mutate(Microbe = str_replace(Microbe, ".*_or_", ""))

# Change the colour for the rare taxa to gray to make them stand out
cols = metafolio::gg_color_hue(length(unique(barlong$Microbe)))
cols[unique(barlong$Microbe)="Rare Taxa"]="dark gray"

# Create the stacked barplots using ggplot2
barlong %>%
  ggplot(aes(x = ID, y = value, fill = Microbe)) +
  geom_bar(stat = "identity", col = "black", size = .2, width = 1) +
  facet_row(~Group, scales = "free_x") +
  # Adjust layout and appearance
  scale_fill_manual(values = cols, labels = unique(sub(".*ales_", ",", barlong$Microbe))) +
  scale_y_continuous(expand = c(0, 0)) +
  guides(fill = guide_legend(ncol = 1, keyheight = 1, title = "Legend")) +
  theme_bw() + xlab("") + ylab("Proportion") +
  theme(text = element_text(size = 14), axis.text.x = element_blank())
```
Stacked barplots are helpful because they allow us to *eyeball* the data, giving us an idea of the experimental setup in the case of more complex designs. They also allow us to get a general sense of the effects we may expect and about the general levels of variance within and between groups. In this case, nothing in particular stands out. These samples look like they could have come from human microbiome sequencing, which is exactly what we want!
2. Alpha Diversity

Another staple in microbiome research is alpha diversity. In a nutshell, alpha diversity is a set of measures that comment on how diverse, complicated and/or rich a single sample is. The three most common metrics for alpha diversity in microbiome research are Chao1, the Simpson Index and Shannon Entropy.

Code chunk: Computing and plotting Alpha diversity from a count table

```r
# It is important to use the untouched count table here as we're interested in rare taxa.
# Compute alpha diversity using a wrapper around the iNEXT library, which implements automatic rarefaction curves.
# This step can take some time.
alpha_diversity = get_asymptotic_alpha(species = counts, verbose = FALSE)

# Add metadata for plotting and stats. Make sure the count table and metadata match up!
alpha_diversity$Legend = metadata$Group
alpha_diversity$Sex = metadata$Sex
alpha_diversity$Smoker = metadata$Smoker

# Plot alpha diversity all at once using pipes
alpha_diversity %>%
  # Wrangle the data to long format for easy plotting
  pivot_longer(!c(Legend, Sex, Smoker)) %>%
  # Pipe it all directly into ggplot2
  ggplot(aes(x = Legend,
             y = value,
             fill = Legend,
             shape = Sex,
             group = Legend)) +
  geom_boxplot(alpha = 1/2, coef = 100) +
  geom_beeswarm(size = 4, cex = 3) +
  facet_wrap(~name, scales = "free") +
  theme_bw() +
  scale_fill_manual(values = c("healthy" = "#fe9929",
                             "schizophrenia" = "#8c6bb1")) +
  scale_shape_manual(values = c("female" = 21,
                                 "male" = 22)) +
  ylab("") + xlab("")
```

When eyeballing these figures, we might be able to spot an effect of both sex and group. Note how in the Chao1 metric, the female healthy volunteers have a much higher diversity than the males. Nevertheless, after taking this sex effect into account, there is still seems to be an effect of group. Rather than relying on eyeballing though, statistical testing will help us determine what to say about these results.

2.1 Testing for differences in Alpha diversity

Usually, one would use standard statistical tests such as t-tests and ANOVAs to assess differences in alpha diversity metrics. However, since we expect that smoking status and sex will also influence the microbiome, we will here opt for a linear model approach to try and account for the effect of sex and smoking status. We will generate tables based on the fitting of these models. When interpreting these tables, we are typically mainly interested in the Estimate column, which is an estimate of the $\beta$ (beta), and the Pr(>|t|) column, which essentially depicts the p-values. In this case, the beta of a group can be readily interpreted as the difference between the means between the respective groups. Since we’ll be estimating a 95% confidence interval as well, We’ll also get two columns with the 2.5% and 97.5% points of the $\beta$ estimate. The top row, containing the (Intercept) gives an estimation of the overall mean of the data and can usually be ignored altogether, don’t get too excited if this has a low p-value.

2.2 Reporting statistical models

According to the STROBE guidelines for observational studies, we should report both the unadjusted model and the adjusted model, it is best practice to do so. We will do so here in the alpha diversity section, but for the sake of brevity we will skip this step for beta diversity and differential abundance. In microbiome studies we typically assess a very large number of features. Comprehensive statistical tables for feature-wise tests will often find a home in the supplementary files.
Code chunk: Testing for differences in Alpha diversity: Chao1

```r
# Fit an unadjusted linear model
cchoa1_unadj = lm(Chao1 ~ Legend, alpha_diversity)

# Combine the summary of the model with the 95% confidence interval of the estimates
res_chao1_unadj = cbind(coefficients(summary(chao1_unadj)), confint(chao1_unadj))

# Plot the unadjusted results in a nice looking table
kable(res_chao1_unadj, digits = 3)
```

|                | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 %  | 97.5 % |
|----------------|----------|------------|---------|---------|--------|--------|
| (Intercept)    | 48.383   | 0.981      | 49.298  | 0.000   | 46.445 | 50.320 |
| Legendschizophrenia | 3.084   | 1.353      | 2.280   | 0.024   | 0.413  | 5.755  |

```r
# Fit a linear model with sex and smoking status as covariates
cchoa1_adj = lm(Chao1 ~ Legend + Sex + Smoker, alpha_diversity)

# Combine the summary of the model with the 95% confidence interval of the estimates
res_chao1_adj = cbind(coefficients(summary(chao1_adj)), confint(chao1_adj))

# Plot the adjusted results in a nice looking table
kable(res_chao1_adj, digits = 3)
```

|                | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 %  | 97.5 % |
|----------------|----------|------------|---------|---------|--------|--------|
| (Intercept)    | 49.649   | 1.190      | 41.723  | 0.000   | 47.300 | 51.999 |
| Legendschizophrenia | 3.012   | 1.350      | 2.231   | 0.027   | 0.347  | 5.677  |
| Sexmale        | -3.067   | 1.520      | -2.018  | 0.045   | -6.067 | -0.066 |
| Smokeryes      | 1.157    | 1.686      | 0.686   | 0.494   | -2.172 | 4.487  |

Code chunk: Testing for differences in Alpha diversity: Shannon Entropy

```r
# Fit an unadjusted linear model
shannon_unadj = lm(`Shannon Entropy` ~ Legend, alpha_diversity)

# Combine the summary of the model with the 95% confidence interval of the estimates
res_shannon_unadj = cbind(coefficients(summary(shannon_unadj)), confint(shannon_unadj))

# Plot the unadjusted results in a nice looking table
kable(res_shannon_unadj, digits = 3)
```

|                | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 %  | 97.5 % |
|----------------|----------|------------|---------|---------|--------|--------|
| (Intercept)    | 1.498    | 0.069      | 21.728  | 0.000   | 1.362  | 1.634  |
| Legendschizophrenia | 0.188   | 0.095      | 1.974   | 0.05    | 0.000  | 0.375  |

```r
# Fit a linear model with sex and smoking status as covariates
shannon_adj = lm(`Shannon Entropy` ~ Legend + Sex + Smoker, alpha_diversity)

# Combine the summary of the model with the 95% confidence interval of the estimates
```

8
res_shann_adj = cbind(coefficients(summary(shann_adj)), confint(shann_adj))

#Plot the adjusted results in a nice looking table
kable(res_shann_adj, digits = 3)

|              | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 % | 97.5 % |
|--------------|----------|------------|---------|----------|-------|--------|
| (Intercept)  | 1.567    | 0.084      | 18.633  | 0.000    | 1.401 | 1.734  |
| Legend        | 0.189    | 0.095      | 1.986   | 0.049    | 0.001 | 0.378  |
| Sex           | -0.128   | 0.107      | -1.195  | 0.234    | -0.341| 0.084  |
| Smoker        | -0.017   | 0.119      | -0.142  | 0.887    | -0.252| 0.218  |

Code chunk: Testing for differences in Alpha diversity: Simpson Index

#Fit an unadjusted linear model
simps_unadj = lm(`Simpson Index` ~ Legend, alpha_diversity)

#Combine the summary of the model with the 95% confidence interval of the estimates
res_simps_unadj = cbind(coefficients(summary(simps_unadj)), confint(simps_unadj))

#Plot the unadjusted results in a nice looking table
kable(res_simps_unadj, digits = 3)

|              | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 % | 97.5 % |
|--------------|----------|------------|---------|----------|-------|--------|
| (Intercept)  | 0.550    | 0.024      | 23.247  | 0.000    | 0.504 | 0.597  |
| Legend        | 0.063    | 0.033      | 1.924   | 0.056    | -0.002| 0.127  |

#Fit a linear model with sex and smoking status as covariates
simps_adj = lm(`Simpson Index` ~ Legend + Sex + Smoker, alpha_diversity)

#Combine the summary of the model with the 95% confidence interval of the estimates
res_simps_adj = cbind(coefficients(summary(simps_adj)), confint(simps_adj))

#Plot the adjusted results in a nice looking table
kable(res_simps_adj, digits = 3)

|              | Estimate | Std. Error | t value | Pr(>|t|) | 2.5 % | 97.5 % |
|--------------|----------|------------|---------|----------|-------|--------|
| (Intercept)  | 0.576    | 0.029      | 19.986  | 0.000    | 0.519 | 0.633  |
| Legend        | 0.063    | 0.033      | 1.939   | 0.054    | -0.001| 0.128  |
| Sex           | -0.049   | 0.037      | -1.332  | 0.185    | -0.122| 0.024  |
| Smoker        | -0.005   | 0.041      | -0.127  | 0.899    | -0.086| 0.076  |
3. Beta Diversity

Beta diversity can be thought of as the degree of difference between two samples. Typically, Beta diversity is depicted using a 2d Principal Component Analysis (PCA). We’ll perform the procedure and visualize the results in a few different ways.

Code chunk: Performing a Principal Component Analysis and plotting Beta diversity

```r
#Apply the base R principal component analysis function on our CLR-transformed data.
data.a.pca <- prcomp(t(genus.exp))

#Extract the amount of variance the first four components explain for plotting.
pc1 <- round(data.a.pca$sdev[1]^2/sum(data.a.pca$sdev^2),4) * 100
pc2 <- round(data.a.pca$sdev[2]^2/sum(data.a.pca$sdev^2),4) * 100
pc3 <- round(data.a.pca$sdev[3]^2/sum(data.a.pca$sdev^2),4) * 100
pc4 <- round(data.a.pca$sdev[4]^2/sum(data.a.pca$sdev^2),4) * 100

#Extract the scores for every sample for the first four components for plotting.
pca = data.frame(PC1 = data.a.pca$x[,1],
    PC2 = data.a.pca$x[,2],
    PC3 = data.a.pca$x[,3],
    PC4 = data.a.pca$x[,4])

#Add relevant information from the metadata
pca$ID = metadata$master_ID
pca$Legend = metadata$Group
pca$Sex = metadata$Sex
pca$Smoker = metadata$Smoker

#First, the main plot. Plot the first two components of the PCA
mainbeta <- ggplot(pca, aes(x = PC1, y = PC2, fill = Legend, colour = Legend, shape = Sex, group = Legend)) +
    stat_ellipse(geom = "polygon", alpha = 1/4) +
    geom_point(size=3, col = "black") +
    scale_fill_manual(values = c("healthy" = "#fe9929", "schizophrenia" = "#8c6bb1")) +
    scale_colour_manual(values = c("healthy" = "#fe9929", "schizophrenia" = "#8c6bb1")) +
    scale_shape_manual(values = c("female" = 21, "male" = 22), guide = "none") +
    guides(fill = guide_legend(override.aes = list(shape = c(21)))) +
    ggtitle("Main") +
    xlab(paste("PC1: ", pc1, "\%", sep="")) +
    ylab(paste("PC2: ", pc2, "\%", sep="")) +
    theme_bw()

#Second, a smaller version to investigate the effect of sex.
```
# Plot the first two components of the PCA

```r
sexbeta <- ggplot(pca, aes(x = PC1, y = PC2, 
fill = Sex, colour = Sex, shape = Sex, 
group = Sex)) +

# Create the points
stat_ellipse(geom = "polygon", alpha = 1/4) + geom_point(size = 2, col = "black") +

# Adjust appearance
scale_fill_manual(values = c("female" = "#fb9a99", "male" = "#a6cee3")) +
scale_colour_manual(values = c("female" = "#fb9a99", "male" = "#a6cee3")) +
scale_shape_manual(values = c("female" = 21, "male" = 22)) +

# Adjust labels
ggtitle("Sex") + xlab("") + ylab("") + theme_bw()
```

# Third, a smaller version to investigate the effect of smoking.

# Plot the first two components of the PCA

```r
smokebeta <- ggplot(pca, aes(x = PC1, y = PC2, 
fill = Smoker, colour = Smoker, shape = Sex, 
group = Smoker)) +

# Create the points
stat_ellipse(geom = "polygon", alpha = 1/4) + geom_point(size = 2, col = "black") +

# Adjust appearance
scale_fill_manual(values = c("yes" = "#ff7f00", "no" = "#33a02c")) +
scale_colour_manual(values = c("yes" = "#ff7f00", "no" = "#33a02c")) +
scale_shape_manual(values = c("female" = 21, "male" = 22), guide = "none") +
guides(fill = guide_legend(override.aes = list(shape = c(21)))) +

# Adjust labels
ggtitle("Smoker status") + xlab("") + ylab("") + theme_bw()
```

# Use patchwork to compose the three plots

```r
(mainbeta / (sexbeta | smokebeta)) +
plot_layout(guides = "collect", heights = c(3, 1))
```
Here we see the first two components of our Principal Component Analysis. This type of figure is often used as a visual aid when assessing Beta diversity. We’ve also made two additional smaller versions of the same data, filled in with our two covariates; sex and smoking status, to help get an idea of the influence of those factors on our data.

Even though we are only looking at the first, largest, two components, this type of figure will often be called a Beta diversity plot. In this case, as we used CLR-transformed data as a basis, it would be based on Aitchison distance. Interpreting a Beta diversity plot for a microbiome study like this one can seem daunting, but will quickly become fairly straightforward. In a nutshell, every sample is depicted as a single point. If two points are close together, this means that the samples are more similar to each other. We can see that group, smoking status and sex seem to be important in explaining what’s going on here.

These types of Beta diversity plots are also a useful way to detect samples that are in some way off. If a sample is on the far side of the PCA, this may be reason to inspect it further. Based on the amount of reads and alpha diversity of the sample, one may even decide to exclude it from the analysis as it may not reliably reflect your population of interest.

Another thing to be on the lookout for are the axis values, depicting the percentage of variance explained. Components that either explain a huge amount of variance or large differences between the amount of variance explained between the first two components can be an indication something drastic is going on, like an antibiotics effect. Typically, we expect the sizes of the components to follow a power law. In this case, the axes look totally reasonable.
3.1 PERMANOVA

We can use a PERMANOVA test to investigate whether the variance in the data can be explained by the

group and sex they come from. Typically, we’d say that we use a PERMANOVA to see whether the groups

are different from each other.

Code chunk: Performing a PERMANOVA test

```r
#Display NAs as empty space in the table to improve appearance.
options(knitr.kable.NA = "")

#Compute euclidean distance over CLR-transformed values (i.e. Aitchison distance).
dis_ait = dist(t(genus.exp), method = "euclidean")

#Perform a PERMANOVA (PERmutational Multivariate ANalysis Of VAriance) test.
PERMANOVA_res = adonis2(dis_ait ~ Group + Sex + Smoker,
                        data = metadata, method = "euclidean", permutations = 1000)

#Plot the PERMANOVA results in a nice looking table
kable(PERMANOVA_res, digits = 4)
```

|     | Df | SumOfSqs | R2    | F      | Pr( > F) |
|-----|----|----------|-------|--------|----------|
| Group | 1  | 872.4806 | 0.0138 | 2.3900 | 0.002    |
| Sex   | 1  | 721.8148 | 0.0114 | 1.9773 | 0.011    |
| Smoker | 1  | 792.3537 | 0.0125 | 2.1705 | 0.003    |
| Residual | 167 | 60963.1982 | 0.9623 |        |          |
| Total | 170 | 63349.8473 | 1.0000 |        |          |

In general, the most interesting columns from a PERMANOVA table like this one are \( R^2 \), which shows the

amount of variance that can be explained by the factor in that row, and \( Pr( > F) \), which can be thought of

as a p-value. We can see that the group but also the smoking status and sex factors explain enough variance

that we deem it unlikely to have happened by chance (\( p < 0.05 \)). Thus, we can say we found a group effect, a

smoking effect and a sex effect. The effect are really small though, both of them explain about 1% of all

variance observed which isn’t very much. This also tracks with our figures, where we could see only mild

differences for each factor. Importantly, because the PERMANOVA is a permutation-based test, all test

results will likely vary slightly between runs. You could use the `set.seed()` function to ensure consistency

between runs, like we did in chapter 0.
4. Differential Abundance

Differential abundance testing is an integral part of microbiome studies. Here we check whether individual features, be they taxa or functions, are present in different abundances between groups.

4.1 Genera

Differential abundance of taxa, in this case genera, are perhaps the most common part of a microbiome study.

Code chunk: Testing for differentially abundant genera and plotting the results

```r
#This function fits the equivalent of lm(feature ~ Group + Sex + Smoker) for each feature. #It also performs an appropriate Benjamini-Hochberg correction on the p-values.

genus.glm = fw_glm(x = genus.exp,
                  f = ~ Group + Sex + Smoker,
                  metadata = metadata,
                  adjust.method = "BH", format = "brief")

# [1] "Using the following formula: x ~ Group + Sex + Smoker"
# [1] "Adjusting for FDR using Benjamini & Hochberg's procedure."

#(genus.glm, file = "genus.glm.csv") #To save the results to a file.
```

Before we proceed with the demonstration, let’s take a quick peek at the output of this function:

```r
glimpse(genus.glm)
```

## Rows: 84
## Columns: 10

```r
## $ feature <chr> "Acidaminococcaceae_Phascolarctobacteriaceae"
## $ Groupschizophrenia Estimate <dbl> 0.38645474, 0.23365778, 1.72428829, 0.81238581, 0.23365778, 0.81238581, 0.81238581, 0.81238581
## $ Groupschizophrenia Pr(>|t|) <dbl> 3.290809e-01, 2.766298e-01, 4.349376e-01, 6.586975e-01, 2.766298e-01, 4.349376e-01, 4.349376e-01, 4.349376e-01
## $ Sexmale Estimate <dbl> 0.485194237, 0.181234364, -0.32451485, 0.181234364, -0.32451485, 0.181234364, -0.32451485, 0.181234364
## $ Sexmale Pr(>|t|) <dbl> 0.2766350, 0.4531579, 0.9547110, 0.9547110, 0.9547110, 0.9547110, 0.9547110, 0.9547110
## $ Smokeryes Estimate <dbl> -0.18867208, 0.65548364, 0.02917598, -0.18867208, 0.65548364, 0.02917598, -0.18867208, 0.65548364
## $ Smokeryes Pr(>|t|) <dbl> 0.70254279, 0.01527056, 0.9547110, 0.9547110, 0.01527056, 0.9547110, 0.9547110, 0.9547110
## $ Groupschizophrenia Pr(>|t|).BH <dbl> 0.537383182, 0.523552585, 0.003653476, 0.003653476, 0.003653476, 0.003653476, 0.003653476, 0.003653476
## $ Sexmale Pr(>|t|).BH <dbl> 0.7524988, 0.8275057, 0.8417461, 0.8417461, 0.8417461, 0.8417461, 0.8417461, 0.8417461
## $ Smokeryes Pr(>|t|).BH <dbl> 0.8930102, 0.1483272, 0.9547110, 0.9547110, 0.1483272, 0.9547110, 0.9547110, 0.9547110
```

The output is a data.frame with the input features as rows and the estimates of betas, p-values and adjusted p-values as columns. This is all direct output from the lm() fits that were run under the hood. If we hadn’t specified format = "brief", we’d also receive the standard error of the estimate and the corresponding t-statistic used to calculate a p-value. It is always a good idea to investigate the output of any bioinformatics pipeline.

Now, let’s proceed with the demonstration.
It is best practice to investigate the distribution of p-values using a histogram.

Histories of p-values are useful to assess whether there is something fishy going on in your data. Broadly speaking, one would typically expect some features to be altered based on a condition (null hypothesis is false; \( H_1 \)) and some others to not be affected by the condition (null hypothesis is true; \( H_0 \)). The p-value was designed in such a way that in the case of a true \( H_0 \), the p-values will be uniformly distributed from 0 - 1. Conversely, in the case of \( H_1 \), the p-values will typically aggregate close to 0. Normally, we would expect a combination of these two patterns in our histogram. So we would want to see a low density of p-values form 0 - 1 with a nice peak around 0 indicating some differences between our groups. This also means that if the p-value histogram is ever overly ‘lumpy’ at a value other than 0, this is an indication that something fishy is going on and that you should try to find out why this is the case. Sometimes, this can happen when using one-tailed tests or when the individual features are heavily dependent on each other.

In this case, the p-value distribution looks fine. We can see that there is a peak on the left. There are many p-values under 0.05. There is a group effect here.
Check the distribution of Benjamini-Hochberg corrected p-values to get a sense of the results. This is not strictly necessary, but it can be helpful to get a sense of how your p-values were affected by the post-hoc correction and how many figures to expect.

```r
hist(genus.glm$'Group schizophrenia Pr(|t|).BH', xlim = c(0, 1), breaks = 20)
```

Using a fairly standard cutoff of $q < 0.1$ we see a fair amount of significant differences.
Plot the features that show a group effect at q < 0.1

```r
genBH <- genus.exp[genus.glm[genus.glm$`Groups: schizophrenia` Pr(|t|).BH < 0.1, "feature", ]
```

```r
genBH %>%
t() %>%
as.data.frame() %>%
add_column( Group = metadata$Group,
            Sex = metadata$Sex ) %>%
pivot_longer(ic("Group", "Sex")) %>%
mutable( name = str_replace( name, ".*ales_", "" ) ) %>%
ggplot( aes( x = Group,
           y = value,
           fill = Group,
           shape = Sex,
           group = Group )) +
  geom_boxplot( alpha = 1/2, coef = 100 ) +
  geom_beeswarm( size = 3, cex = 3 ) +
  facet_wrap(~ name, scales = "free_y", ncol = 4 ) +
  scale_fill_manual( values = c( "healthy" = "#fe9929",
                                "schizophrenia" = "#8c6bb1" ) ) +
  scale_shape_manual( values = c( "female" = 21,
                                 "male" = 22 ) ) +
  ylab("") + xlab("") + theme_bw() + theme( text = element_text( size = 12 ) )
```

It seems eight genera are significantly differential abundant between our healthy and schizophrenia groups given the q < 0.1 threshold. Note that the y-axis depicts CLR-transformed abundance.

#write.csv(genus.glm, "genus.glm.csv") #To save the results to a file.
4.2 Functional Modules

Functional modules provide many advantages over taxa when it comes to differential abundance analysis. Perhaps prime among them, they are easier to interpret as they cover concrete molecular processes rather than the abundance of a microbe that may not be characterized.

Code chunk: Load the Gut Brain Modules and prepare them for analysis

```r
#Ensure reproducibility within this document
set.seed(1)

#Load GBMS like we did with the genus-level counts and metadata above.
GBMs <- GBMs

#Make sure our count data is all numbers
GBMs <- apply(GBMs,c(1,2),function(x) as.numeric(as.character(x)))

#Remove features with prevalence < 10% in two steps:
#First, determine how often every feature is absent in a sample
n_zeroes_GBMs <- rowSums(GBMs == 0)

#Then, remove features that are absent in more than your threshold (90% in this case).
GBMs <- GBMs[n_zeroes_GBMs <= round(ncol(GBMs) * 0.90),]

#Perform a CLR transformation
GBMs.exp <- clr_c(GBMs)

#Reorder the CLR-transformed feature table to match the metadata
GBMs.exp = GBMs.exp[,metadata$master_ID]

#This function fits the equivalent of lm(feature ~ Group + Sex + Smoker) for each feature.
#It also performs an appropriate Benjamini-Hochberg correction on the p-values.
GBMs.glm = fw_glm(x = GBMs.exp,
                   f = ~ Group + Sex + Smoker,
                   metadata = metadata,
                   adjust.method = "BH")
```

## [1] "Using the following formula: x ~ Group + Sex + Smoker"
## [1] "Adjusting for FDR using Benjamini & Hochberg's procedure."
It is best practice to investigate the distribution of p-values using a histogram.

```r
hist(GBMs.glm$'Groupschizophrenia Pr(>|t|)', xlim = c(0, 1), breaks = 20)
```

Histograms of p-values are useful to assess whether there is something fishy going on in your data. Broadly speaking, one would typically expect some features to be altered based on a condition (null hypothesis is false; $H_1$) and some others to not be affected by the condition (null hypothesis is true; $H_0$). The p-value was designed in such a way that in the case of a true $H_0$, the p-values will be uniformly distributed from 0 - 1. Conversely, in the case of $H_1$, the p-values will typically aggregate close to 0. Normally, we would expect a combination of these two patterns in our histogram. So we would want to see a low density of p-values form 0 - 1 with a nice peak around 0 indicating some differences between our groups. This also means that if the p-value histogram is ever overly ‘lumpy’ at a value other than 0, this is an indication that something fishy is going on and that you should try to find out why this is the case. Sometimes, this can happen when using one-tailed tests or when the individual features are heavily dependent on each other.

In this case, the p-value distribution looks fine. We can see that there is a peak on the left. There are many p-values under 0.05. There is a group effect here.
Check the distribution of Benjamini-Hochberg corrected p-values to get a sense of the results. This is not strictly necessary, but it can be helpful to get a sense of how your p-values were affected by the post-hoc correction and how many figures to expect.

\[
\text{hist(GBMs.glm$'Groupschizophrenia Pr(|t|).BH', xla lim = c(0, 1), breaks = 20)}
\]

Using a fairly standard cutoff of \(q < 0.2\) we see a few hits.
Code chunk: Plot the differentially abundant Gut Brain modules

```r
#Plot the features that show a group effect at q < 0.2
GBM_BH <- GBMs.exp[GBMs.glm[GBMs.glm$Groupschizophrenia Pr>|t|.BH` < 0.2,"feature"],]

GBM_BH %>%
t() %>%
as.data.frame() %>%
add_column(Group = metadata$Group,
  Sex = metadata$Sex) %>%
pivot_longer(!c("Group", "Sex")) %>%
mutate(name = str_replace(name, ".*ales_", "")) %>%
ggplot(aes(x = Group,
  y = value,
  fill = Group,
  shape = Sex,
  group = Group)) +
geom_boxplot(alpha = 1/2, coef = 100) +
geom_beeswarm(size = 3, cex = 3) +
facet_wrap(~name, scales = "free_y", ncol = 3) +
scale_fill_manual(values = c("healthy" = "#fe9929",
  "schizophrenia" = "#8c6bb1")) +
scale_shape_manual(values = c("female" = 21,
  "male" = 22)) +
ylab("") + xlab("") + theme_bw() + theme(text = element_text(size = 12))

#write.csv(GBMs.glm, "GBMs.glm.csv") #To save the results to a file.
```
# Ensure reproducibility within this document
set.seed(1)

# Load GBMS like we did with the genus-level counts and metadata above.
GMMs <- GMMs

# Make sure our count data is all numbers
GMMs <- apply(GMMs, c(1, 2), function(x) as.numeric(as.character(x)))

# Remove features with prevalence < 10% in two steps:
# First, determine how often every feature is absent in a sample
n_zeroes_GMMs <- rowSums(GMMs == 0)

# Then, remove features that are absent in more than your threshold (90% in this case).
GMMs <- GMMs[n_zeroes_GMMs <= round(ncol(GMMs) * 0.90),]

# Perform a CLR transformation
GMMs.exp <- clr_c(GMMs)

# Reorder the CLR-transformed feature table to match the metadata
GMMs.exp <- GMMs.exp[, metadata$master_ID]

# This function fits the equivalent of lm(feature ~ Group + Sex + Smoker) for each feature.
# It also performs an appropriate Benjamini-Hochberg correction on the p-values.
GMMs.glm = fw_glm(x = GMMs.exp,
                   f = ~ Group + Sex + Smoker,
                   metadata = metadata,
                   adjust.method = "BH")

## [1] "Using the following formula: x ~ Group + Sex + Smoker"
## [1] "Adjusting for FDR using Benjamini & Hochberg's procedure."
It is best practice to investigate the distribution of p-values using a histogram.

```r
hist(GMMs$'Groupschizophrenia Pr(>|t|)', xlim = c(0, 1), breaks = 20)
```

Histograms of p-values are useful to assess whether there is something fishy going on in your data. Broadly speaking, one would typically expect some features to be altered based on a condition (null hypothesis is false; $H_1$) and some others to not be affected by the condition (null hypothesis is true; $H_0$). The p-value was designed in such a way that in the case of a true $H_0$, the p-values will be uniformly distributed from 0 - 1. Conversely, in the case of $H_1$, the p-values will typically aggregate close to 0. Normally, we would expect a combination of these two patterns in our histogram. So we would want to see a low density of p-values form 0 - 1 with a nice peak around 0 indicating some differences between our groups. This also means that if the p-value histogram is ever overly ‘lumpy’ at a value other than 0, this is an indication that something fishy is going on and that you should try to find out why this is the case. Sometimes, this can happen when using one-tailed tests or when the individual features are heavily dependent on each other.

In this case, the p-value distribution looks fine. We can see that there is a peak on the left. There are many p-values under 0.05. There is a group effect here.
Check the distribution of Benjamini-Hochberg corrected p-values to get a sense of the results. This is not strictly necessary, but it can be helpful to get a sense of how your p-values were affected by the post-hoc correction and how many figures to expect.

```r
hist(GMMs$Groupschizophrenia Pr(>|t|).BH, xlim = c(0, 1), breaks = 20)
```

Using a fairly standard cutoff of $q < 0.2$ we see a fair amount of significant differences.
#Plot the features that show a group effect at \( q < 0.2 \)

\[
\text{GMM\_BH} \leftarrow \text{GMMs.exp}[\text{GMMs.glm}[\text{GMMs.glm}^\text{Grpschizophrenia Pr(>|t|).BH} < 0.2, \text{"feature"}],]
\]

\[
\text{GMM\_BH} \gg
\text{t()} \gg
\text{as.data.frame()} \gg
\text{add_column(} \text{Group = metadata}\$\text{Group,}
\text{Sex = metadata}\$\text{Sex}) \gg
\text{pivot\_longer(}!\text{c("Group", "Sex")}) \gg
\text{mutate(name = str\_replace(name, ".*ales\_", ",")}) \gg
\text{ggplot(aes(x = Group,}
\text{y = value,}
\text{fill = Group,}
\text{shape = Sex,}
\text{group = Group))} +
\text{geom\_boxplot(alpha = 1/2, coef = 100) +
geom\_beeswarm(size = 3, cex = 3) +
facet\_wrap(~name, scales = \text{"free\_y"}, ncol = 4) +
scale\_fill\_manual( values = c("healthy" = \"#fe9929",
\"schizophrenia" = \"#8c6bb1\") +
scale\_shape\_manual(values = c("female" = 21,
\"male" = 22)) +
ylab("") + xlab("") + theme\_bw() + theme(text = element\_text(size = 12))
\]
#write.csv(GMMs.glm, "GMMs.glm.csv") #To save the results to a file.
5. Discussion

Here, we have presented what a fairly standard microbiome analysis might look like. The main points we would take from the analysis would be that there is indeed a difference in the microbiome between our cohort of patients with schizophrenia and healthy volunteers, in terms of composition (beta diversity), diversity (alpha diversity) as well as in differential feature abundance, both on the taxonomical level as well as the functional level. We could then go on and comment on what specific functions in the microbiome may explain the differences in our cohort. For instance, differences in the metabolism of DOPAC and glutamate, precursor molecules for the important neurotransmitters dopamine and GABA, could be pointed out and compared to literature. In our limitations section, we would stress that the effect sizes we found were quite small and that we found an effect of smoking and of sex as well.

Of course, this document is just a template. Depending on the experimental setup, findings and experimental questions, you may want to choose a differing approach. Given the highly complex nature of microbiome data, one should ideally avoid blindly applying models and pipelines without understanding what they are doing. D.R. Cox is famously ascribed the statement: “Most real life statistical problems have one or more nonstandard features. There are no routine statistical questions; only questionable statistical routines.” We find this holds true for the microbiome as well.

Clear communication, both in terms of describing and explaining our methods as well as in terms of figure presentation, are essential for the health of the field. Indeed, failing to do so can lead to confusion among our peers. We hope that both aspiring and veteran bioinformaticians will find our guide helpful. We have tried to model this piece after what we would have loved to have access to ourselves when we first set out to study the microbiome.
Excursion 1. Computing functional modules

Functional modules such as the Gut Brain modules shown here can be a valuable framework to investigate your data through. One major benefit of this framework is that you greatly reduce your search-space to specific functional pathways you're interested in. This will allow you to greatly save on statistical tests, which in turn will help save statistical power when accounting for FDR.

Here, we will demonstrate how we got to the Gut Brain modules from our the schizophrenia data set. Be warned that the first few operations here are computationally very expensive and should be performed on a server or similar if possible.

Gathering and preparing our data

First, let’s download the necessary data from curatedMicrobiomeData

```r
#Load the relevant libraries
library(curatedMetagenomicData)
library(SummarizedExperiment)

#Define what data we're interested in. The "|" sign here signifies that we want both.
query <- "2021-03-31.ZhuF_2020.relative_abundance|2021-03-31.ZhuF_2020.gene_families"

#Download the specified data. This will take time.
ZhuF <- curatedMetagenomicData(query, counts = T, dryrun = F)

#Extract the relevant data from complex SummarizedExperiment objects
Zhu_F_gene_families = as.matrix(SummarizedExperiment::assay(ZhuF[[1]]))
Zhu_F_microbiome = SummarizedExperiment::assay(ZhuF[[2]])
Zhu_F_metadata = data.frame(SummarizedExperiment::colData(ZhuF[[2]]))

#Now that we have our data, we can write the individual tables to csv files.
#We'll focus on the gene families here, they're necessary to compute Gut Brain modules.
write.csv(Zhu_F_gene_families, file = "uniref90.csv")
```

Convert to KEGG orthologues

Zhu_F_gene_families contains the functional microbiome in terms of uniref90. For functional module analysis, we typically want to get to KEGG orthologues (KOs). Because curatedMetagenomicData gives essentially biobakery output, we can use commands from the python-based HUMAnN3 pipeline to translate our uniref90 table to KEGG orthologues. We will not go deeply into this, but see the excellent documentation here: https://github.com/biobakery/humann#humann_regroup_table

The next snippet is not R code, but rather Bash code.

```bash
#First, we may want to change our uniref90 file so that it uses tabs instead of commas
sed -E 's/\(["{}\]*\)\?\|/\1\2\|/g' uniref90.csv > uniref90.tsv

#Then, let's use humann_regroup_table from HUMAnN3 to convert to KEGG orthologues:
humann_regroup_table -i uniref90.tsv -g uniref90_ko -o guidebook_out_KOs.tsv
```
Compute functional modules

Now that we have our functional microbiome in terms of KEGG orthologues, we can load them back into R. An added benefit is that the KEGG orthologues table is much smaller than the uniref90 table, so we can deal with it on our computer locally if we so choose. In order to do this, we will require the omixer-rpmR library which can be found of github. If you’re working with functional inference data from a 16S experiment, such as output from PICRUSt2, you should be able to read the table in and compute functional modules starting at this step. The file you’re looking for would be called something like pred_metagenome_unstrat.tsv in that case.

```r
#Load the required package
library(omixerRpm) #devtools::install_github("omixer/omixer-rpmR")

#Load the KEGG orthologue table into R.
#Note that KEGG orthologue names should be the in first column, not the row names.
KOs <- read.delim("guidebook_out_KOs.tsv", header = T)

#listDB will tell you which databases are available to annotate the functional data with.
listDB()

#Pick the most recent GBM database
db <- loadDB(name = "GBMs.v1.0")

#Calculate GBM abundance and convert the output to a nice data.frame
GBMs <- rpm(x = KOs, module.db = db)
GBMs <- asDataFrame(GBMs, "abundance")

#Write the file to a csv to save it.
write.csv(GBMs, file = "GBMs_guidebook.csv")

#While we’re at it, let’s do GMMs too: First check the names of the available databases:
listDB()

#Pick the most recent GMM database
db <- loadDB(name = "GMMs.v1.07")

#Calculate GMM abundance and convert the output to a nice data.frame
GMMs <- rpm(x = KOs, module.db = db)
GMMs <- asDataFrame(GMMs, "abundance")

#Write the file to a csv to save it.
write.csv(GMMs, file = "GMMs_guidebook.csv")

And the resulting files are ready for statistical analysis! I would like to note here that it’s also possible to perform a stratified functional module analysis, where the contribution of each taxon to each functional module is also considered. However, this explosively increases the dimensionality of your data (i.e. you get way more rows in our case). I would only recommend doing this as a targeted analysis as the power of any statistical tests will suffer greatly and the results will be almost impossible to interpret.
Excursion 2. Volatility Analysis

Volatility refers to the degree of instability (or change over time) in the microbiome. High volatility, i.e. an unstable microbiome, has been associated with an exaggerated stress response and conditions like IBS. Here, we’ll demonstrate how one would go about calculating volatility in a real data set. Volatility analysis requires at least two time points per sample. Because of this, we cannot use the schizophrenia data set which only features single snapshots of the microbiome. (Hence this excursion.)

We’ll be taking a look at the datasets used in the original Volatility paper: *Volatility as a Concept to Understand the Impact of Stress on the Microbiome* (DOI: 10.1016/j.psyneuen.2020.105047). Very briefly, mice were separated into two groups: Control and Stress. Faecal samples were taken twice, with a 10-day period in between. In this 10-day period, the mice in the Stress group were subjected to daily social defeat stress, whereas the Control mice were left alone. When we compared the degree of change in the microbiome (i.e. Volatility) between the two groups of mice, the Stressed mice consistently displayed higher levels of volatility than the control mice. Our reviewers asked us to replicate the experiment and we did so. The two cohorts are labeled discovery and validation. This data has been included in the volatility library on github for easy access purposes.

Traditionally, microbiome studies featuring high-throughput sequencing data only consider a single time point. However, there is utility in considering microbiomes as dynamic microbial ecosystems that change over time. By measuring the microbiome longitudinally and computing volatility, additional information can be revealed that would otherwise be missed. For instance, in the original volatility study, we found that volatility after stress is positively associated with severity of the stress response, including in terms of behaviour and hypothalamic-pituitary-adrenal (HPA) axis activity, both in mice and in humans.

Setup

OK, now let’s get started.

```r
#Install and load volatility library
library(volatility)  #devtools::install_github("thomazbastiaanssen/volatility")

#Load tidyverse to wrangle and plot results.
library(tidyverse)

#Load example data + metadata from the volatility study.
data(volatility_data)
```


Considering our input data

The main volatility function does all the heavy lifting here. It expects two arguments: The first argument is counts, a microbiome feature count table, with columns as samples and rows and features. The vola_genus_table object is an example of an appropriately formatted count table.

```r
vola_genus_table[4:9,1:2]
##  
## Atopobiaceae_Oscenella 0 0
## Coriobacteriaceae_Collinsella 0 0
## Eggerthellaceae_DNF00809 102 47
## Eggerthellaceae_Enterorhabdus 53 114
## Eggerthellaceae_Parvibacter 21 20
## Bacteroidaceae_Bacteroides 616 453
```

The second argument is metadata, a vector in the same order as the count table, denoting which samples are from the same source. The column ID in vola_metadata is appropriate for this.

```r
head(vola_metadata, 5)
##   sample_ID  cohort timepoint treatment ID
## 1 Validation_Pre_Control_1 Validation Pre Control 1
## 2 Validation_Pre_Control_2 Validation Pre Control 2
## 3 Validation_Pre_Control_3 Validation Pre Control 3
## 4 Validation_Pre_Control_4 Validation Pre Control 4
## 5 Validation_Pre_Control_5 Validation Pre Control 5
```

Prepare the data for plotting

```r
# This part should feel very reminiscent of what we did in chapters 1 & 3.
counts <- vola_genus_table[,vola_metadata$sample_ID]

# Fork off your count data so that you always have an untouched version handy.
genus <- counts

# Make sure our count data is all numbers
ngenus <- apply(genus, c(1,2), function(x) as.numeric(as.character(x)))

# Remove features with prevalence < 10% in two steps:
# First, determine how often every feature is absent in a sample
n_zeroses <- rowSums(ngenus == 0)

# Then, remove features that are absent in more than your threshold (90% in this case).
genus <- genus[n_zeroses <= round(ncol(genus) * 0.90),]

# Perform a CLR transformation
genus.exp <- clr_c(genus)

# Apply the base R principal component analysis function on our CLR-transformed data.
data.a.pca <- prcomp(t(genus.exp))
```
Plot longitudinal PCA

# Extract the amount of variance the first four components explain for plotting.
p1 <- round(data.a.pca$sdev[1]^2/sum(data.a.pca$sdev^2),4) * 100
p2 <- round(data.a.pca$sdev[2]^2/sum(data.a.pca$sdev^2),4) * 100
p3 <- round(data.a.pca$sdev[3]^2/sum(data.a.pca$sdev^2),4) * 100
p4 <- round(data.a.pca$sdev[4]^2/sum(data.a.pca$sdev^2),4) * 100

# Extract the scores for every sample for the first four components for plotting.
pca = data.frame(PC1 = data.a.pca$x[,1],
                 PC2 = data.a.pca$x[,2],
                 PC3 = data.a.pca$x[,3],
                 PC4 = data.a.pca$x[,4])

# Add relevant information from the metadata.
# Note that ID here refers to the mouse ID, not the sample ID.
pca$ID = vola_metadata$ID
pca$Legend = vola_metadata$treatment
pca$Timepoint = vola_metadata$timepoint
pca$Cohort = vola_metadata$cohort

# Plot the first two components of the PCA.
ggplot(pca, aes(x = PC1,
                y = PC2,
                fill = Legend,
                colour = Legend,
                shape = Timepoint,
                group = ID)) +
  geom_line() +
  geom_point(size = 3, col = "black") +

# Plot the two cohorts separately.
facet_wrap(~Cohort, scales = "free_x", strip.position = "top") +

# Improve appearance.
scale_fill_manual( values = c("Control" = "#1f78b4",
                                "Stress" = "#e31a1c")) +
scale_colour_manual(values = c("Control" = "#3690c0",
                                "Stress" = "#cb181d")) +
scale_shape_manual( values = c("Pre" = 21,
                                "Post" = 24)) +
theme_bw() +
xlab(paste("PC1: ", p1, ", p", sep="")) +
ylab(paste("PC2: ", p2, ", p", sep="")) +
theme(text = element_text(size = 12)) +
guides(fill = guide_legend(override.aes = list(shape = 22)))
We can see that points from the same mouse are connected by a line. It looks like some lines are longer than others, implying that some microbiomes have changed more than others over the 10 days. However, We’re only looking at about 30% of the variance here, so it’s hard to say anything conclusive.

**Compute volatility**

Volatility between two samples can be easily calculated using the titular `volatility` function in the library by the same name. Under the hood, volatility can be calculated as the euclidean distance over CLR-transformed count data.

```r
vola_out <- volatility(counts = genus, metadata = vola_metadata$ID)
head(vola_out)
```

```
## ID volatility
## 1 1 13.275363
## 2 10 12.979717
## 3 11 15.987468
## 4 13 14.902772
## 5 14 8.094823
## 6 15 9.506299
```

The output of the main `volatility` function is a data.frame with two columns. `ID` corresponds to the pairs of samples passed on in the `metadata` argument, whereas `volatility` shows the measured volatility between those samples in terms of Aitchison distance (Euclidean distance of CLR-transformed counts).
Plot the results

```r
vola_out %>%
  # Merge the volatility output with the rest of the metadata using the shared "ID" column.
  left_join(vola_metadata[vola_metadata$timepoint == "Pre"], "ID") %>%

  # Pipe into ggplot.
  ggplot(aes(x = treatment, y = volatility, fill = treatment)) +

  # Define geoms, boxplots overlayed with data points in this case.
  geom_boxplot(alpha = 1/2, coef = 10) +
  geom_point(shape = 21) +

  # Split the plot by cohort.
  facet_wrap(~cohort) +

  # Tweak appearance.
  scale_fill_manual(values = c("Control" = "#3690c0", "Stress" = "#cb181d")) +
  theme_bw() +
  xlab("") +
  ylab("Volatility (Aitchison distance)")
```

![Graph showing volatility distribution for Discovery and Validation datasets with comparisons between Control and Stress groups.](image-url)