Microbiome data analysis & visualization

Systems Medicine in Maternal and Early Life (BIO514)

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Australian National Phenome Centre

CSM

Murdoch University

Welcome

Acknowledgement of country

"I acknowledge that Murdoch University is situated on the lands of the Whadjuk Noongar people.

I pay respect to their enduring and dynamic culture and the leadership of Noongar elders both past and present.

The boodjar (country) on which Murdoch University is located has for thousands of years, been a place of learning. We at Murdoch University are proud to continue this long tradition."

Outline

Workshops and tutorials on microbiome and genomics

Today we will be doing through some microbiome bioinformatics.

Workshop notes and data available:

GitHub code repository `siobhon-egan/BIO514-microbiome`
Website with information & tutorials `siobhonlegan.com/BIO514-microbiome`

Workflow

1. Wet lab
2. Microbiome bioinformatics
   - Set up
   - Sequence processing
   - Data cleaning
   - Data visualization

Disclaimer ⚠

I don't consider myself to be a primarily a "coder". My scientific background and training has largely been in areas of biology, infectious disease (parasitology) and molecular biology/genomics. I become a bioinformatician out of need to analyse the data I generated in the lab.

My advice is to think of the world of coding as someone learning microsoft office for the first time.

1. You do not have to be an expert. Jump in and give it a go. Start with the basics and expand from there.
2. You'll pick up what you need to know as you go along. Similar to above don't expect to be a professional at the start. Things will be messy but do whatever works for you!
3. There are lots of ways to do the same thing. Just because what is on the person next to you screen doesn't mean what you have (or what they have is wrong).
4. Google is your friend. First thing is just copy and paste error message into google. Forums like stack overflow will likely have your answer.
5. Document everything you do! Find a way that works for you. I like `Rmarkdown` files, but to start with try just using a word/google doc to keep a record of your code.

References

- Johnson, J.S., Spakowicz, D.J., Hong, BY. et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun 10, 5029 (2019). doi: 10.1038/s41467-019-13036-1
- Pollock J, Glendinning L, Wisedchanwet T, Watson M. The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. Appl Environ Microbiol. 2018;84(7):e02627-17. doi: 10.1128/AEM.02627-17
- Nilakanta H, Drews KL, Firrell S, Foulkes MA, Jablonski KA. A review of software for analyzing molecular sequences. BMC Res Notes. 2014;7:830. doi: 10.1186/1756-0500-7-830
- Roumpeka DD, Wallace RJ, Escalettes F, Fotheringham I, Watson M. A Review of Bioinformatics Tools for Bio-Prospecting from Metagenomic Sequence Data. Front Genet. 2017;8:23. doi: 10.3389/fgene.2017.00023
- Schriefer AE, Cliften PF, Hibberd MC, Sawyer C, Brown-Kennerly V, Burcea L, Klotz E, Crosby SD, Gordon JI, Head RD. A multi-amplicon 16S rRNA sequencing and analysis method for improved taxonomic profiling of bacterial communities. J Microbiol Methods. 2018;154:6-13. doi: 10.1016/j.mimet.2018.09.019
- Bharti R, Grimm DG. Current challenges and best-practice protocols for microbiome analysis. Brief Bioinform. 2021;22(1):178-193. doi: 10.1093/bib/bbz155
- Allaband C, McDonald D, Vázquez-Baeza Y, et al. Microbiome 101: Studying, Analyzing, and Interpreting Gut Microbiome Data for Clinicians. Clin Gastroenterol Hepatol. 2019;17(2):218-230. doi: 10.1016/j.cgh.2018.09.017
- Lepage P, Leclerc MC, Joossens M, et al. A metagenomic insight into our gut's microbiome. Gut 2013;62:146-158. doi: 10.1136/gutjnl-2011-301805
- Ref: Liu, YX., Qin, Y., Chen, T. et al. A practical guide to amplicon and metagenomic analysis of microbiome data. Protein Cell (2020). 10.1007/s13238-020-00724-8

Glossary

- **Microbiome**
    - The microorganisms of a specific habitat and surrounding environment. Sometimes specific for bacteria, but also can be used more broadly for microscopic organisms (e.g. viruses, single-cell eukaryotes, bacteria and sometimes parasites).
- **Metagenomics**
    - All the genetic material recovered directly from environmental samples.
- **OTUs**
    - Operation taxonomic units. Generally considered to be clustered at 97% similar level - species level.
- **ASVs**
    - Amplicon sequence variants. Denoised sequence variants. Equivalent to zero radius operational taxonomic units (zOTU).
- **16S**
    - 16S ribosomal RNA gene, small subunit of a prokaryotic ribosome (SSU rRNA).
- **Hypervariable region**
    - Portions in the genome of a taxa with much higher levels of variation than other similar areas.

Glossary

- **Cluster**
  - Algorithms that attempt to group related biological sequences, generally at a set threshold, for example: species level = 97% (e.g. OTUs).
- **Denoise**
  - A computational method for removing sequence errors and identifying correct true biological sequences in the reads. These approaches provide improved resolution and result in unique biological sequences (e.g. ASVs, ZOTUs).
- **OTU table**
  - Also known as count data, contains the list of OTU/ASVs and number of sequences per sample.
    In this example each row is a sample and a column is the OTU/ASV.
- **Taxonomy table**
  - Spreadsheet containing OTU/ASV and taxonomic identify, generally as 7 columns (Kingdom, Phylum, Class, Order, Family, Genus, Species).
- **Sample data**
  - contains metadata associated with samples.
- **Phyloseq object**
  - Multi-component data set merging OTU table, taxonomy table, sample data, sequences and phylogenetic table. Part of the phyloseq R package.

Setup

REQUIRED

We will be using RStudio to analyse the data set. It is recommend you have the following installed: *RStudio version 1.4* or later and *R version 4.0* or later. Further details on getting started in RStudio here.

Optional (*not needed for today's workshop*)

We will not be doing the sequence pre-processing steps today but if you did want to do this you will need to download conda and QIIME2.

> If you are you are interested in genomic bioinformatics try install/set up this in the breaks or come make a time to see me if any issues.

Setup

- **Sequence data** (*optional*)

  Raw amplicon 16S sequence data from West et al. (2020) *Gut* 69, 1452-1459. doi: 10.1136/gutjnl-2019-319620. Download raw data from NCBI Sequence Read Archive. Project number `PRJNA493625` from https://sra-explorer.info/.

  You will not be required to download this for today's tutorial but if you wanted you could use this data and follow the sequence processing page.

I have also uploaded pre-processed QIIME2 sequence data as outlined in sequence processing. This is available for download on FigShare. Download files and you can view them using QIIME2 view.

Setup

- **RData** (*REQUIRED*)

  The easiest way to follow along with this tutorial is to download this GitHub repository using either option **1** or **2** below:

  1. Go to https://github.com/siobhon-egan/BIO514-microbiome and click on the green **Code** button. Select **Download ZIP**, open/unzip the file. Open the `.Rmd` files in RStudio you will be able to follow along for the data analysis.
  2. Use terminal and clone the GitHub repo.

  ```
  git clone https://github.com/siobhon-egan/BIO514-microbiome.git
  ```

In the **data/** directory you will find:

- Three `.csv` files which contain the output from the QIIME2 pipeline. The files are: (1) otu_tabe (count data), (2) tax_table (taxonomy) and (3) sam_data (sample meatdata).
- An `.Rdata` file which we will load into R for the analysis - really this is just a file that contains all three of the spreadsheets above in an R format that is already formatted and ready to go for analysis.

Methods

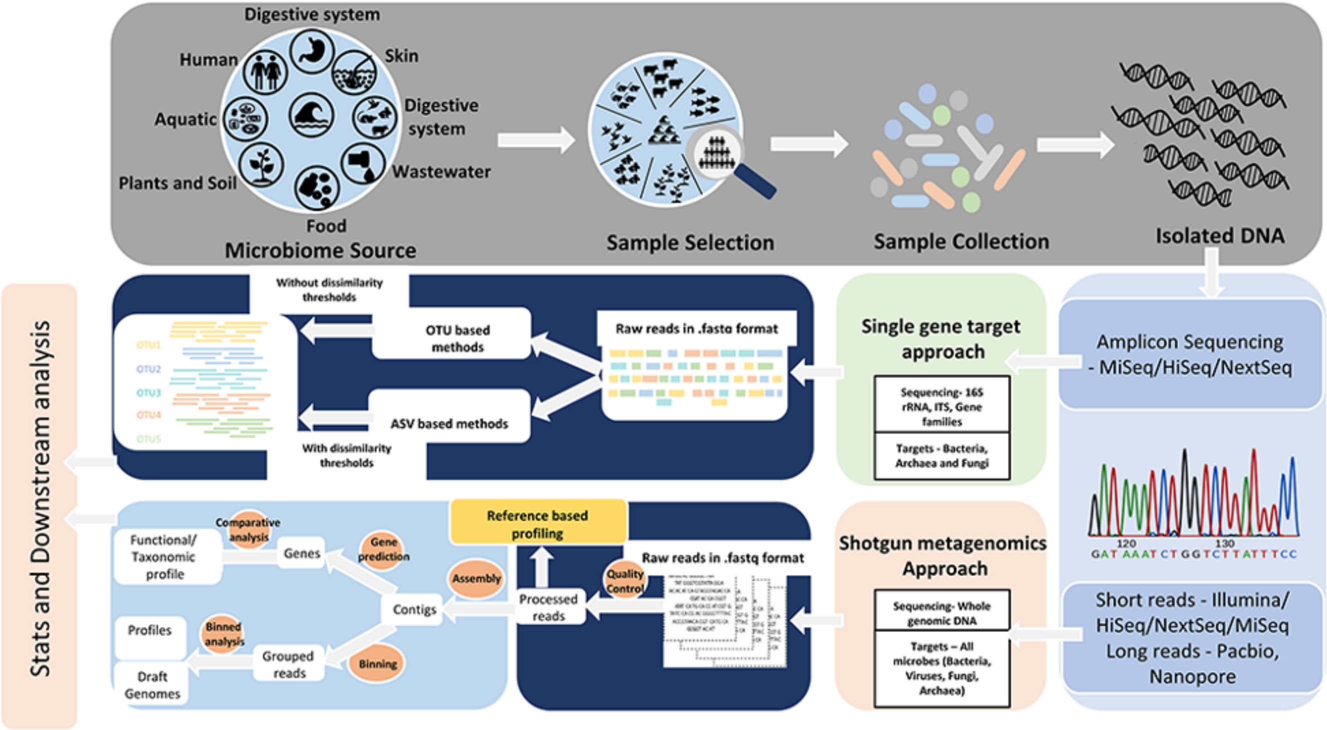
Exert direct from West et al. 2020. *Gut*. doi: 10.1136/gutjnl-2019-319620.

Stool samples were randomised for processing and DNA was extracted (see online supplementary methods) using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio). 16S rRNA gene amplicon sequencing targeting the V1-V2 regions was performed on the Illumina MiSeq platform as previously described[21]. Raw reads were processed in the R software environmentt[19] following a published workflow[22] which includes amplicon denoising implemented in 'DADA2'[23]. See (online supplementary methods) for full details. Functions in the 'vegan' R package were used to calculate Shannon Diversity Indices (alpha-diversity) on data rarefied to the minimum sequencing depth and Bray-Curtis dissimilarity (beta-diversity) on log-transformed data (pseudocount of 1 added to each value). Significance of group separation in beta-diversity was assessed by permutational multivariate analysis of variance. Changes in relative abundance were tested at each taxonomic rank from phylum to genus using the Mann-Whitney U test while differentially abundant 16S rRNA gene sequences were identified using 'DESeq2'[24]. For 'DESeq2' analysis, data were pooled for each individual rather than analysing distinct time points.

[19] R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2017. https://www.R-project.org/. [21] Mullish BH , Pechlivanis A , Barker GF , et al . Functional microbiomics: evaluation of gut microbiota-bile acid metabolism interactions in health and disease. Methods 2018;149:49–58. doi:10.1016/j.ymeth.2018.04.028. [22] Callahan BJ , Sankaran K , Fukuyama JA , et al . Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. Version 2. F1000Res 2016;5:1492. doi:10.12688/f1000research.8986.1 [23] Callahan BJ , McMurdie PJ , Rosen MJ , et al . DADA2: high-resolution sample inference from illumina amplicon data. Nat Methods 2016;13:581–3. doi:10.1038/nmeth.3869 [24] Love MI , Huber W , Anders S . Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. Genome Biol 2014;15:550. doi:10.1186/s13059-014-0550-8

Background

Microbiome, metagenomics and bioinformatics is a huge area of study so we certainly wont be covering all aspects of it here.


Digestive system
Human
Skin
Aquatic
Digestive system
Plants and Soil
Wastewater
Food
Microbiome Source
Sample Selection
Sample Collection
Isolated DNA
Without dissimilarity thresholds
OTU based methods
Raw reads in .fasta format
OTU1
OTU2
OTU3
OTU4
OTU5
ASV based methods
With dissimilarity thresholds
Single gene target approach
Sequencing- 16S rRNA, ITS, Gene families
Targets - Bacteria, Archaea and Fungi
Amplicon Sequencing - MiSeq/HiSeq/NextSeq
120
130
GAT AAAT CT GGTCTTATTTCC
Stats and Downstream analysis
Comparative analysis
Functional/ Taxonomic profile
Genes
Gene prediction
Reference based profiling
Raw reads in .fastq format
Assembly
Quality Control
Contigs
Processed reads
Profiles
Binned analysis
Grouped reads
Binning
Draft Genomes
Shotgun metagenomics Approach
Sequencing- Whole genomic DNA
Targets – All microbes (Bacteria, Viruses, Fungi, Archaea)
Short reads - Illumina/ HiSeq/NextSeq/MiSeq Long reads - Pacbio, Nanopore

Targeted amplicon and metagenomic sequencing approaches[1].

[1] Ref: Bharti And Grimm (2019) *Briefings in Bioinformatics* 22(1) doi: 10.1093/bib/bbz155.

Today there are two main **molecular** approaches that we use for microbiome studies.

1. Metagenomics = DNA

2. Metatranscriptomics = messenger RNA

Metagenomics = DNA

- Genomic characterisation of bacteria.
- Identify what bacteria is present in sample.
- Further broken down into
  - Amplicon sequencing
  - Shotgun/whole genome sequencing

Amplicon 16S rRNA sequencing.

- Sequence the 16S rRNA gene (targeting bacteria only).
- Use primers targeting the 16S gene - hypervariable regions (V1-9).
- There are bias/differences between primers and regions.
- Ref: Bukin, Y., Galachyants, Y., Morozov, I. et al. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data 6, 190007 (2019). doi: 10.1038/sdata.2019.7
- More recent advances in "long-read" platforms (e.g. PacBio, nanopore) allow for full length 16S rRNA gene sequences.
- Currently not widely used but this will quickly change as technology becomes more widely available.
- Ref: Johnson, J.S., Spakowicz, D.J., Hong, BY. et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun 10, 5029 (2019). doi: 10.1038/s41467-019-13036-1

Shotgun/whole genome sequencing

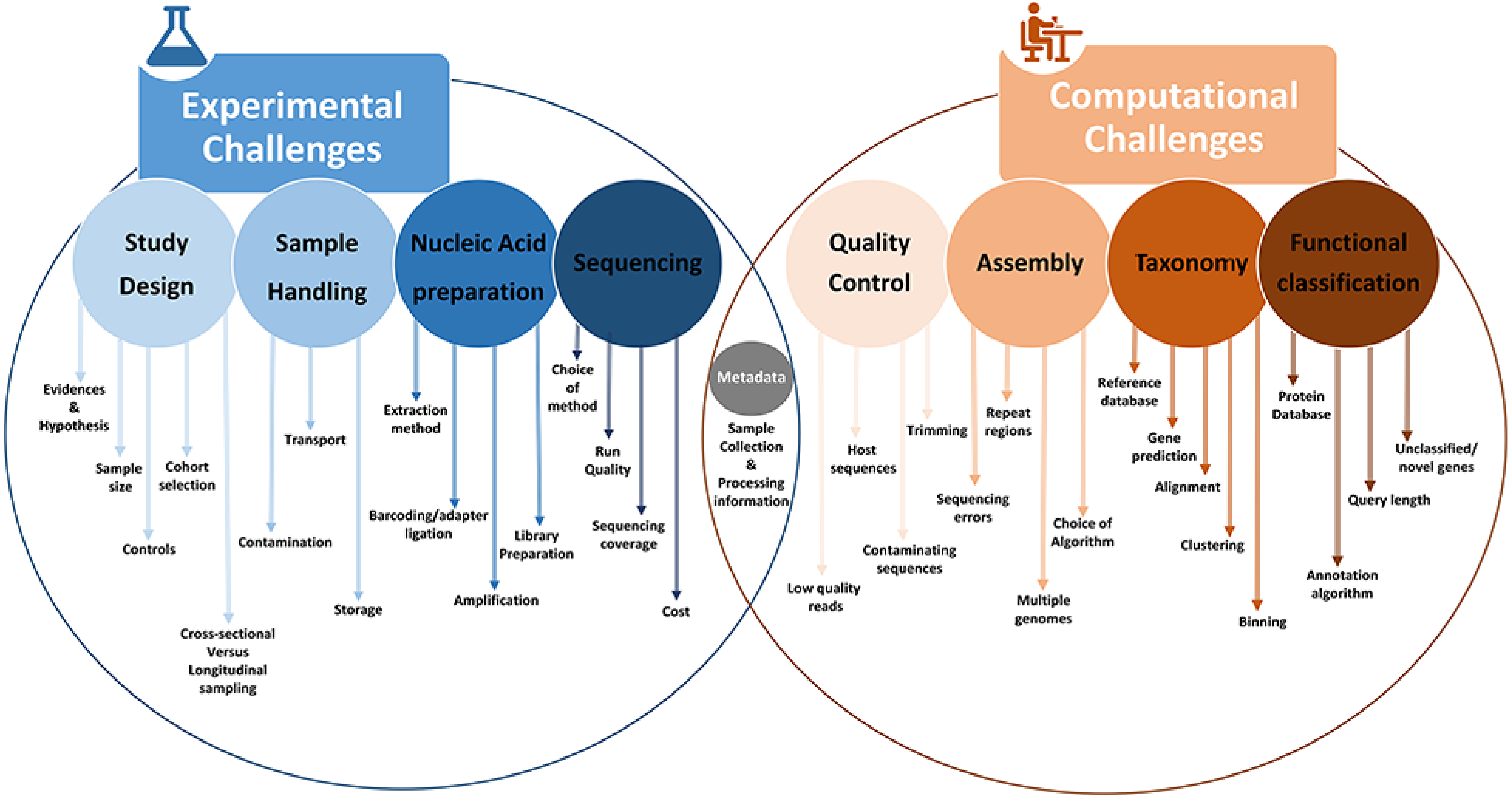
- Sequence all the genomic material within the sample.
- This will include the host (e.g. human) DNA as well so need much deeper level of sequencing.
- Able to sequence viral communities - extract RNA and convert to cDNA.

Pros of amplicon over shotgun

- Cheaper
- Less data intensive
- Easier to make sense of...e.g. good reference databases available.
- More sensitive at detecting lower abundant bacteria (shot gun sequencing = mainly host DNA)

Metatranscriptomics = messenger RNA

- Gene expression and regulation
- Used for functional potential
- Better for relative abundance comparison - no PCR bias



A schematic overview outlining various experimental and computational challenges associated with 16S rRNA-based and shotgun metagenomic sequencing[1].

[1] Bharti And Grimm (2019) *Briefings in Bioinformatics* 22(1) doi: 10.1093/bib/bbz155.

Terminology note

- You may see reference to difference sequencing platforms when you read so just to clarify. Next-generation sequencing = high throughput sequencing. Although now terminology has moved to "short-read" vs "long-read" sequencing. But when reading most articles next-generation sequencing usually equals short read sequencing.
- Short read platforms
    - 454 - pyrosequencing
    - Ion Torrent - semiconductor sequencing
    - Illumina - clusters on flow cell (most common)
        - Machines: iSeq NextSeq (300 bp), MiniSeq NextSeq (300 bp), MiSeq (max 600 bp), NextSeq (300 bp), Nova Seq (500 bp)
- Long read platforms - technologies still developing to improve accuracy
    - PacBio
    - Nanopore

Bioinformatics

We will only briefly go through these steps to give you an idea of what is involved. There are various programs and databases required for these steps - so you won't be performing all of these on your machines today.

Instead I'll go through the main steps and give you access to some scripts. Then I'll share with you the output files that we will use for the data visualization part.

There is a wealth of information and different pipelines available but generally most use very similar algorithms *under the hood*.

Sequence Processing

Main steps of processing 16S amplicon sequencing

1. Demultiplex
2. Merge, trim and filter
3. Cluster & denoise
4. Assign taxonomy

The most widely used pipelines include:

- USEARCH - either UPARSE or UNOISE
- dada2
- Mouthur
- vsearch
- QIIME2 - this using either dada2 or vsearch

Step 1. Demulitplex

- Use of barcodes (i.e. sequence of 6-8 nucleotides added to primers to identify individual samples).
- Depending on library prep used and sequencing platform this might be automated.
- E.g. Illumina and Nextera indexes are automatically demultiplexed on sequencing machine.

Step 2. Merge, Trim & Filter

Merge - *optional*

Depending on sequence platform/pipeline if you have forward and reverse reads you may first need to merge these. Most pipelines have built in merge function so you can avoid using a separate program. In the case of QIIME2 you **do not** need to merge reads. This step is fairly straight forward and not much difference between programs. PEAR is a popular stand alone program.

Trim

Depending on pipeline this can be done along side filtering.

- Lots of options available, again I try and keep number of programs etc to a minimum. Most pipelines will have some sort of trimming/QC function built in.
- FASTQC is popular for viewing sequence files and automating QC reports.

Filter

Comments as above. Depending on your samples and design you may need more stringent filtering. Many pipelines have additional filtering options i.e. removing low abundant sequences etc.

Step 3. Cluster or denoise

- Group related sequences.
- Traditional approaches relied on *clustering*.
  - Grouped sequences that were within 97% similar i.e group sequences at the species level.
  - Common tools = vsearch (use stand alone or within QIIME2 pipeline) and uparse (used within USEARCH pipeline).
- Newer approaches use *denoising* method.
  - More accurate method to correct sequencing errors and determine real biological sequences at single nucleotide resolution by generating amplicon sequence variants (ASVs).
  - Common tools = dada2 (use stand alone or within QIIME2 pipeline) and unoise3 (used within USEARCH pipeline).

Terminology: The data produced from the clustering/denoising step is referred to a either "Operational Taxonomic Units (OTUs)" or "Amplicon Sequence Variants (ASVs)". Unfortunately terminology in genomics is not always consistent. But as a general rule of thumb OTUs refer to data produced via clustering and ASVs refers to data produced by denoising (however unoise3 in USEARCH refers to these as Zero-radius taxonomic units (ZOTUs) in this case ZOTU = ASV).

Step 4. Assign taxonomy

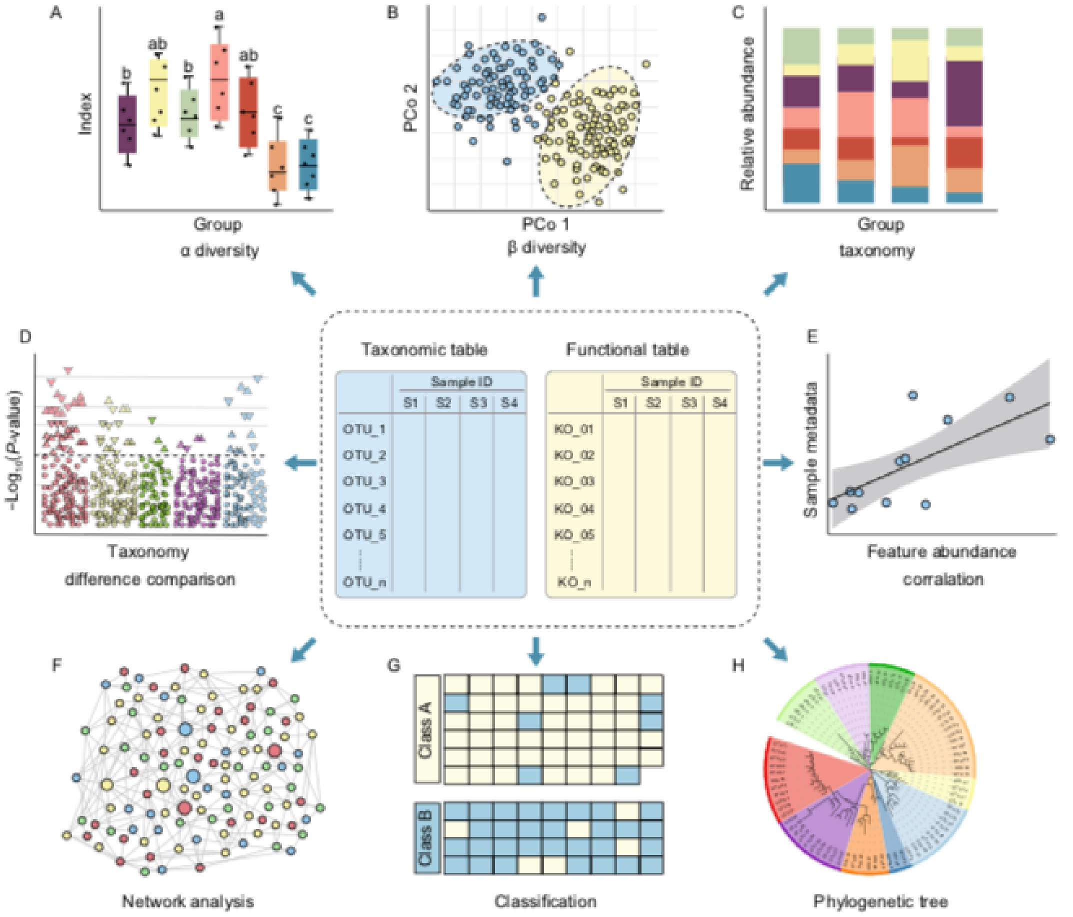
- Algorithms on taxonomic assignment and classification level (e.g. Genus, Family etc). Rarely obtain accurate species level assignment with 16S amplicon but depends on the amplicon region, size, taxa group and region of 16S gene.
    - q2-feature-classifier - used in QIIME2 pipeline (one of the best options currently available).
    - SINTAX - used within USEARCH pipeline.
- Curated databases with representative of taxa. Comparison of main databases - SILVA, RDP, Greengenes, NCBI and OTT how do these taxonomies compare? Balvociute and Huson (2017) BMC Genomics, 18(2), 114. doi: 10.1186/s12864-017-3501-4.
    - Greengenes
    - SILVA
    - RDP

Data cleaning and visualization

There are a number of different analysis and visualization options that you can use depending on your data and questions.

Some common examples include:

- Rarefaction curves
- Alpha diversity plots
- Taxonomy barplots/heatmaps
- Beta diversity and ordination
- Network analysis
- Correlation
- Phylogenetic



Taxonomic table

	Sample ID			
	S1	S2	S3	S4
OTU_1				
OTU_2				
OTU_3				
OTU_4				
OTU_5				
⋮				
OTU_n				

Functional table

	Sample ID			
	S1	S2	S3	S4
KO_01				
KO_02				
KO_03				
KO_04				
KO_05				
⋮				
KO_n				

Overview of statistical and visualization methods for feature tables. Downstream analysis of microbiome feature tables, including alpha/beta-diversity (A/B), taxonomic composition (C), difference comparison (D), correlation analysis (E), network analysis (F), classification of machine learning (G), and phylogenetic tree (H)[1].

[1] Liu, YX., Qin, Y., Chen, T. et al. A practical guide to amplicon and metagenomic analysis of microbiome data. Protein Cell (2020). 10.1007/s13238-020-00724-8.

In this part of the workshop we will go through some different ways you can visualize the data and some statistical analysis. We will do this in RStudio. Just like the bioinformatic sites above there is a wealth of options for this. My personal preference is RStudio as it is easily reproducible (*VERY* important for bioinformatics) and is easy to upscale. In addition with the ever increasing data being produced RStudio provides the best platform to integrate different data types and create custom pipelines.

Working within RStudio environment is not limited to just running code locally on your machine. RShiny allows you to make custom apps and web interface programs..

Further detail on cleaning data after processing sequences is covered here

Links

Useful links for microbial genomics analysis

- Happy Belly Bioinformatics - A useful website containing information, tutorials and links related to bioinformatics (written by a biologist turned bioinformatician!)
- mixOmics - Our mixOmics R package proposes a whole range of multivariate methods that we developed and validated on many biological studies to gain more insight into ‘omics biological studies. Useful GitBook here
- phyloseq - R package for the analysis of microbial communities brings many challenges. Integration of many different types of data with methods from ecology, genetics, phylogenetics, network analysis, visualization and testing
- Tools for Microbiome Analysis - A list of R environment based tools for microbiome data exploration, statistical analysis and visualization
- My own list of useful microbiome resources - this includes some links to RShiny packages which provide an interactive look at your data. However they require your data to be in a specific format.

Sequence Processing example

As mentioned there are lots of options for processing sequence data, this work flow uses the QIIME2 pipeline. While you can also perform statistical analysis and visualize your data in QIIME2, as it is a web based platform it is restricted in terms of analysis options, customising figures, cleaning & subsetting data and integrating other data.

My approach & recommendations:

- Keep up to date with the latest, best practice pipelines and algorithms.
    - However you will need to draw a line at some point.
    - Decide on a method and stick to it.
    - Document what you did and why.
    - **Hint**: this is why good documentation at time of analysis is so important! You *will not* remember in a few days/weeks/months what and why you analysed the data in a certain way.
- Use open source programs
    - Reproducibility - don't have to rely on subscriptions etc.
    - Easier to collaborate and allow others to help you.
    - Good documentation and community forums.
- Minimize the number of different languages/programs required.
    - "Easy-to-use" GUI program may seem promising, but can create down stream issues with integrating other data.
    - As programs/environments get updated, it can limit portability.

You need to find a balance that works for you and your study question(s).

File formats

Before we begin let's just go over some different file format terminology.

FASTQ

- Text-based sequencing data file format that stores both raw sequence data and quality scores.
- FASTQ files have become the standard format for storing NGS data from Illumina sequencing systems, and can be used as input for a wide variety of secondary data analysis solutions.
- Each entry in a FASTQ file consists of 4 lines:
    - Sequence identifier
    - Sequence
    - Quality score identifier line (consisting only of a +)
    - Quality score
- The first line, identifying the sequence, contains the following elements.
    - `@<instrument>:<run number>:<flowcell ID>:<lane>:<tile>:<x-pos>:<y-pos>:<UMI> <read>:<is filtered>:<control number>:<index>`

FASTA

- Very widely used sequence format.
- It consists of a header line starting with a `>` character followed by a code identifying the sequence (and description). The header line is followed by one or more lines containing the sequence itself. FASTA files may contain one or more sequences. open in text editor.

BIOM

- The BIOM file format (canonically pronounced biome) is designed to be a general-use format for representing biological sample by observation contingency tables.
- Handles storage of large, sparse biological contingency tables
- Support encapsulation of core study data (contingency table data and sample/observation metadata) in a single file
- Facilitate the use of these tables between tools that support this format (e.g., passing of data between different programs.).

Trees

Trees can be encoded in a number of different formats, all of which must represent the nested structure of a tree. They may or may not encode branch lengths and other features. Standardized formats are critical for distributing and sharing trees without relying on graphics output that is hard to import into existing software. Commonly used formats are

- nexus
- newick

Spreadsheets containing data

Open in excel/google sheets or text editor

- tab-separated values (TSV)
- comma-separated values (CSV)

R files

- `.R` - R scripts
- `.Rmd` - R markdown file. Contain a mix of text and "code chunks"
- `.RData`or `.rda` - for storing a complete R workspace or selected "objects" from a workspace in a form that can be loaded back by R

QIIME2

Official QIIME2 docs, and view objects via QIIME2 view.

Customised scripts available at https://github.com/siobhon-egan/qiime2_analysis

Pipeline created with QIIME2-2020.11, see QIIME2 documentation for install based on your platform.

QIIME2 introduces its own files formats known as `.qza.` and `.qzv` files. This are unique to QIIME2 and you will likely need to convert these to some other readable format.

Import data

Import .fastq.gz data into QIIME2 format using Casava 1.8 demultiplexed (paired-end) option. Remember assumes raw data is in directory labeled raw_data/ and file naming format as above.

```
qiime tools import \
--type 'SampleData[PairedEndSequencesWithQuality]' \
--input-path raw_data \
--input-format CasavaOneEightSingleLanePerSampleDirFmt \
--output-path 16S_demux_seqs.qza

# create visualization file
qiime demux summarize \
  --i-data 16S_demux_seqs.qza \
  --o-visualization 16S_demux_seqs.qzv
```

Inspect 16S_demux_seqs.qzv artifact for quality scores. This will help decide on QC parameters.

Denoising

Based on quality plot in the above output `16S_demux_seqs.qza` adjust trim length to where quality falls.

Then you can also trim primers. In this case working with 16S V1-2 data.

```
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs 16S_demux_seqs.qza \
   --p-trim-left-f 20 \
   --p-trim-left-r 19 \
   --p-trunc-len-f 250 \
  --p-trunc-len-r 250 \
  --o-table 16S_denoise_table.qza \
  --o-representative-sequences 16S_denoise_rep-seqs.qza \
  --o-denoising-stats 16S_denoise-stats.qza
```

At this stage, you will have artifacts containing the feature table, corresponding feature sequences, and DADA2 denoising stats. You can generate summaries of these as follows.

```
qiime feature-table summarize \
  --i-table 16S_denoise_table.qza \
  --o-visualization 16S_denoise_table.qzv \
  --m-sample-metadata-file sample-metadata.tsv # Can skip this bit if needed.

qiime feature-table tabulate-seqs \
  --i-data 16S_denoise_rep-seqs.qza \
  --o-visualization 16S_denoise_rep-seqs.qzv

qiime metadata tabulate \
  --m-input-file 16S_denoise-stats.qza \
  --o-visualization 16S_denoise-stats.qzv
```

Export ASV table

To produce an ASV table with number of each ASV reads per sample that you can open in excel.

Need to make biom file first

```
qiime tools export \
--input-path 16S_denoise_table.qza \
--output-path feature-table

biom convert \
-i feature-table/feature-table.biom \
-o feature-table/feature-table.tsv \
--to-tsv
```

Phylogeny

Several downstream diversity metrics require that a phylogenetic tree be constructed using the Operational Taxonomic Units (OTUs) or Amplicon Sequence Variants (ASVs) being investigated.

```
qiime phylogeny align-to-tree-mafft-fasttree \
  --i-sequences rep-seqs.qza \
  --o-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza \
  --o-rooted-tree rooted-tree.qza
```

Export

Covert unrooted tree output to newick formatted file

```
qiime tools export \
  --input-path unrooted-tree.qza \
  --output-path exported-tree
```

Taxonomy

Assign taxonomy to denoised sequences using a pre-trained naive bayes classifier and the q2-feature-classifier plugin. Details on how to create a classifier are available here.

I am using a pre-training classifier for the 16S V1-2 with reference a SILVA database version 138.1.

Note that taxonomic classifiers perform best when they are trained based on your specific sample preparation and sequencing parameters, including the primers that were used for amplification and the length of your sequence reads.

Classifier

```
qiime feature-classifier classify-sklearn \
--i-classifier /Taxonomy/QIIME2_classifiers_v2020.11/Silva_99_Otus/27F-388Y/classifier.qza \
--i-reads 16S_denoise_rep-seqs.qza \
--o-classification qiime2-taxa-silva/taxonomy.qza

qiime metadata tabulate \
--m-input-file qiime2-taxa-silva/taxonomy.qza \
--o-visualization qiime2-taxa-silva/taxonomy.qzv
```

Data output

Lets take a took at something prepared earlier.

There are two major outputs from the process above.

```
pkgs <- c("readr", "rmarkdown")
library("DT"); library("dplyr")
lapply(pkgs, require, character.only = TRUE)
otu_table <- read_csv("../data/otu_table.csv", skip = 1, col_names=FALSE)
otu_table <- rename(otu_table, Sample_name = X1)
otu_table_sub <- select(otu_table, Sample_name, X2, X3, X4, X5, X6, X7, X8, X9, X10, X11, X1
```

1. The count data

This is the data contains the list of ASVs and number of sequences per sample.
In this example each row is a sample and a column is the OTU/ASV.

Count data - showing first 20 OTUs only

```
otu_table_sub %>%
  DT::datatable(class = "compact", extensions = "Buttons",
                options = list(dom = 'tBp', buttons = c("csv","excel"),
                               pageLength = 8))
```

	Sample_name	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X1
1	1_4	199	1595	1	24	31	0	19	14	0	4	17	1	50	0	0	
2	10_1	5	1289	0	247	358	0	0	48	110	0	1	6	1	0	1	
3	10_2	4	1434	1	1583	682	0	0	471	41	0	0	12	0	86	0	
4	10_4	204	917	2017	0	1450	398	59	0	93	0	176	0	0	326	13	
5	100_4	2485	34	0	1	1	1386	1102	0	1	0	532	0	0	0	1	
6	11_1	969	359	57	0	314	0	0	0	2	5	127	34	3	0	67	24
7	11_2	1356	487	109	2	438	1	1	14	0	16	154	4	0	0	60	25
8	11_4	748	188	41	0	138	1	1	0	0	3	35	24	0	31	19	25

CSV Excel

Previous 1 2 3 4 5 … 9 Next

Taxonomy

```
df_tax %>%
  DT::datatable(class = "compact", extensions = "Buttons",
                options = list(dom = 'tBp', buttons = c("csv","excel"),
                               pageLength = 8))
```

	Kingdom	Phylum	Class	Order	Family	Genus
1	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
2	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
3	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
4	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Sh
5	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
6	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroide
7	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
8	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Sh

CSV Excel

Previous 1 2 3 4 5 … 540 Next

Data cleaning

While we will not perform these steps today but we'll spend a few minutes just talking about some data processing steps after sequence clustering/denoising.

Depending on your aims the importance of these steps will differ.

Check taxonomy

- Importance will depend on what the aims of your study are.
- For most 'composition' studies or those relying on broad scale trends in patterns this won't be as important.
- The best method/databases will depend on your taxa.
- For some groups of bacteria differentiation using partial sequence of 16S gene is not possible (e.g. *Rickettsia*).
- Dependent on region of 16S gene. Generally sequencing the start (e.g. V1-2) has less references available then the middle of the 16S gene (e.g. V3-4).

Two steps/parameters to consider for taxonomy assignment

1. Assignment algorithms. 2. Reference databases.

Generally pipelines use curated bacteria 16S databases but if your sequence does not match any bacteria it will classify as unknown. Most people use NCBI BLAST against all sequence data to confirm identity for any taxa that require further confirmation. Curated database are used because they provide better accuracy, especially if you have a trained classifier as used in QIIME2. They also take up much less computer space (easier to store).

The more accurate your taxonomic assignment the better downstream analysis is - in particular **functional assignment** and **16S gene copy number prediction** (see below).

Clean sequence count data

Use controls to subtract that "background" bacteria noise.

Additional bonus points for

- Using mock communities (e.g. Mock Bacteria ARchaea Community; MBARC-26, ref: Singer et al. *Sci Data* 3, 160081 (2016). doi: 10.1038/sdata.2016.81)
- Quantifying input DNA concentration which can then be used for frequency detection methods using decontam package (details below).

The `Decontam` R Package

decontam with detailed tutorial

Reference: Davis et al. (2018) *Microbiome*, 6, 226 doi: 10.1186/s40168-018-0605-2.

Simple code for identifying contaminant taxa based on prevalence data. Phyloseq object is `ps` and the sample data has a column called `Sample_or_Control` where the control samples are `Control Sample`. Default prevalence threshold is set to `0.1`. I recommend you check what taxa is identified as "contaminant" and see if it makes sense (this where its good to know something about your samples/microbiology). You may need to adjust threshold as needed, e.g. for more aggressive classification threshold rather than the default try `0.5`.

Define control samples and identify taxa present in them using prevalence method.

```r
sample_data(ps)$is.neg <- sample_data(ps)$Sample_or_Control == "Control Sample"
contamdf.prev <- isContaminant(ps, method="prevalence", neg="is.neg")

# Identify how many contaminants
head(which(contamdf.prev$contaminant))
# Identify what the contaminants are
table(contamdf.prev$contaminant)
```

Set contaminant threshold (default is 0.1).

```
contamdf.prev01 <- isContaminant(ps, method="prevalence", neg="is.neg", threshold=0.1)
table(contamdf.prev01$contaminant)
```

Then you "subtract" these taxa from your data set. Raw data is phyloseq object `ps` and will create new phyloseq object for downstream analysis `ps.decon`

```
ps.decon <- prune_taxa(!contamdf.freq$contaminant, ps)
ps.decon
```

Alternative: a very similar R package called `microDecon` also available GitHub repo.
Reference: McKnight et al. (2019) *Environmental DNA*, 1, 14-25 doi: 10.1002/edn3.11.

Functional assignment

Bacterial profiling based on 16S rRNA-based surveys gives a "who’s there?" answer. However as our knowledge improves more questions arise and now we are moving to answer question about "what can they do?".

Just like with taxonomy databases there are functional databases that group taxa into functional groups. The polypeptides predicted from these sequences are annotated by homology to gene function databases.

A word of caution

"...inference with the default database is likely limited outside of human samples and that development of tools for gene prediction specific to different non-human and environmental samples is warranted." - Quote from Sun et al. (2020) *Microbiome* 8, 45 doi: 10.1186/s40168-020-00815-y

Popular databases:

- PICRUSt - Langille et al. (2013) *Nat Biotechnol* 31(9), 814-821 doi: 10.1038/nbt.2676
- CopyRighter - Angly et al. (2014) *Microbiome* 2, 11 doi: 10.1186/2049-2618-2-11
- PAPRICA - Dowman and Ducklow (2015) 10(8), e0135868 *PLoS ONE* doi: 10.1371/journal.pone.0135868
- Tax4Fun - ABhauer et al (2015) *Bioinformatics.* 31, 2882–4, doi: 10.1093/bioinformatics/btv287

Correct for 16S sequence abundance

Number of copies of the 16S rRNA gene in bacteria varies (1-15). Still not widely used and so far databases/tools are not worthwhile.

Summary of findings in Louca et al. (2018). *Microbiome* 6, 41 doi: 10.1186/s40168-018-0420-9.

- "...16S gene copy numbers (GCNs) could only be accurately predicted for a limited fraction of taxa, namely taxa with closely to moderately related representatives (<15% divergence in the 16S rRNA gene)."
- "...all considered tools exhibit low predictive accuracy when evaluated against completely sequenced genomes, in some cases explaining less than 10% of the variance."
- "Substantial disagreement was also observed between tools (R2<0.5) for the majority of tested microbial communities"
- *In summary*: "We recommend **against correcting for 16S GCNs** in microbiome surveys by default..."

Some other references:

- PICRUSt - Langille et al. (2013) *Nat Biotechnol* 31(9), 814-821 doi: 10.1038/nbt.2676
- CopyRighter - Angly et al. (2014) *Microbiome* 2, 11 doi: 10.1186/2049-2618-2-11
- PAPRICA - Dowman and Ducklow (2015) 10(8), e0135868 *PLoS ONE* doi: 10.1371/journal.pone.0135868
- UNBIAS Edgar preprint available in USEARCH

BREAK

Make sure you have R installed for when we come back.

Data Visualization

This data is `phyloseq` format. This is the most commonly used data format for amplicon data in RStudio. As we have skipped over getting our data into R, here are some help links on this matter phyloseq and customised tutorial here.

Essentially we need at least three bits of data that talk to each other:

- **Count data** - sometimes called OTU data. Usually OTUs/ASVs are rows and each column is sample.
- **Taxonomy data** - this contains the taxonomy of the count (or OTU) data. Each row is a unique OTU/ASV and column reflect **Kingdom, Phylum, Class, Order, Family, Genus, Species**.
- **Sample data** - sometimes referred to as metadata. This includes all the additional information on samples e.g. sample variables such as collection time, patient age, disease status etc.

Optional data

- **Phylogenetic tree** - usually as newick format but other options available. For some beta-diversity analysis this is required
- **Ref sequences** - sequences of OTUs or ASV (as `.fasta` format)

Download GitHub Repo

The easiest way to follow along with this tutorial is to download this GitHub repository using either option **1** or **2** below:

1. Go to https://github.com/siobhon-egan/BIO514-microbiome and click on the green **Code** button. Select **Download ZIP**, open/unzip the file. Open the `.Rmd` files in RStudio you will be able to follow along for the data analysis.

1. Use terminal and clone the GitHub repo.

```
git clone https://github.com/siobhon-egan/BIO514-microbiome.git
```

Load libraries

```
pkgs <- c("tidyverse", "santaR", "phyloseq", "ggpubr", "ggplot2",
          "vegan", "DESeq2", "mixOmics", "Hmisc", "igraph", "ppcor",
          "reshape2", "plotly", "microbiomeutilities", "ampvis2",
          "MicrobiotaProcess", "microbiome", "DirichletMultinomial",
          "magrittr")
lapply(pkgs, require, character.only = TRUE)
```

Load data

Load data in RData - downloaded from https://github.com/ka-west/PBS_manuscript

```
load("../data/PBS_data.Rdata")
# Quick glance at phyloseq object
ps_M
```

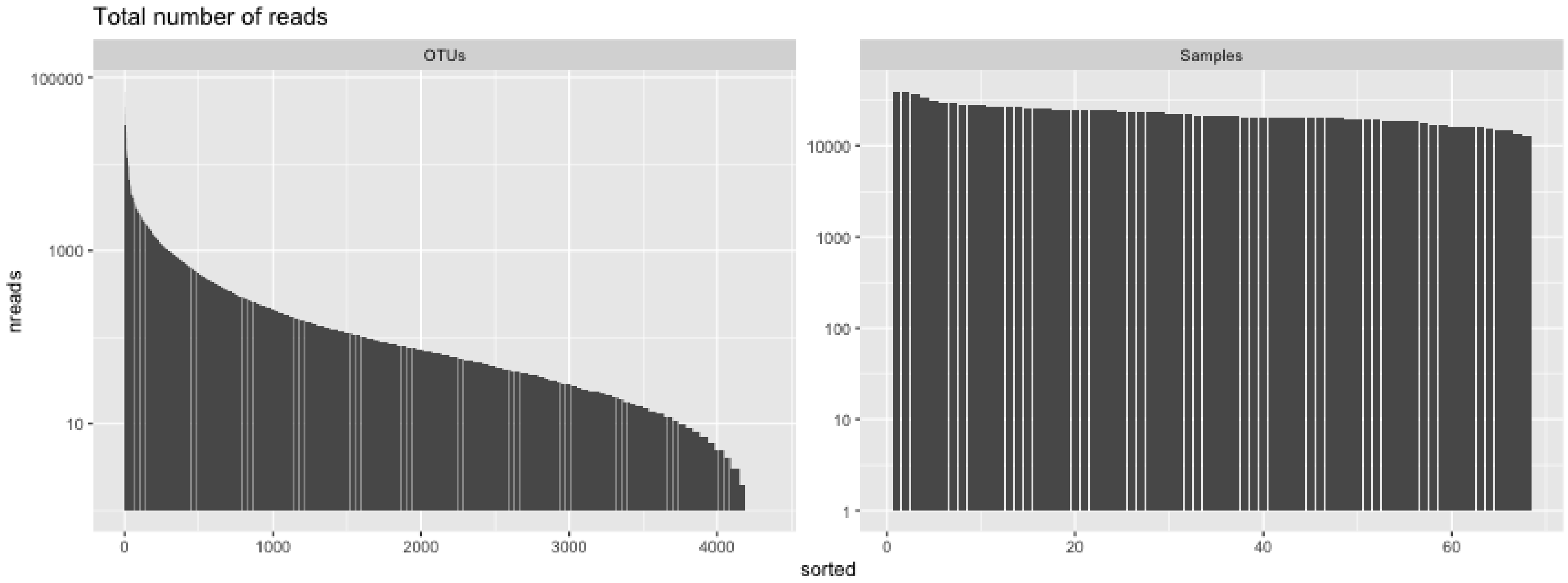
```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 4318 taxa and 68 samples ]
sample_data() Sample Data:       [ 68 samples by 15 sample variables ]
tax_table()   Taxonomy Table:    [ 4318 taxa by 6 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 4318 tips and 4316 internal nodes ]
```

Inspect data

Number of reads

This will give you an overview of the number of reads per sample and per OTU. Important to know the 'depth' of sequencing. Generally for amplicon 16S microbiome you want many 10's of thousands of (good reads) per sample. The more complex the sample the more reads you need (but there is a very large variation in studies and not set rule).

```
readsumsdf = data.frame(nreads = sort(taxa_sums(ps_M), TRUE), sorted = 1:ntaxa(ps_M
    type = "OTUs")
readsumsdf = rbind(readsumsdf, data.frame(nreads = sort(sample_sums(ps_M),
    TRUE), sorted = 1:nsamples(ps_M), type = "Samples"))
title = "Total number of reads"
nreads = ggplot(readsumsdf, aes(x = sorted, y = nreads)) + geom_bar(stat = "identit
nreads = nreads + ggtitle(title) + scale_y_log10() + facet_wrap(~type, 1, scales = '
nreads
```



Read density plot

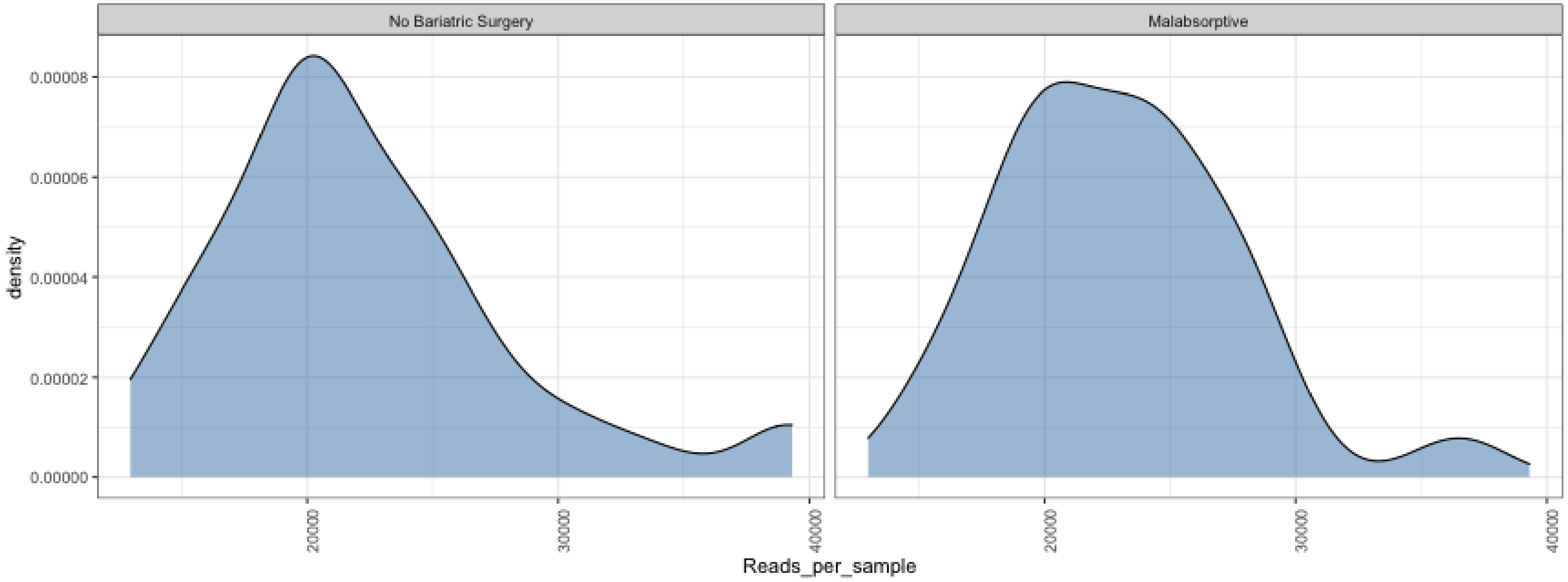
Useful for QC purposes. This will show you the distribution of sequencing depth among samples. Ideally you want an even number of reads per sample. If you see lots of variation then library preparation needs to be optimised and you will need to perform more thorough data cleaning (i.e. rarefy reads - but this is not ideal.

Ref: McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol. 2014 3;10(4):e1003531. doi: https://doi.org/10.1371/journal.pcbi.1003531.

```
read_distrib <- plot_read_distribution(ps_M, groups = "Group",
                                       plot.type = "density")
```

```
[1] "Done plotting"
```

```
read_distrib
```

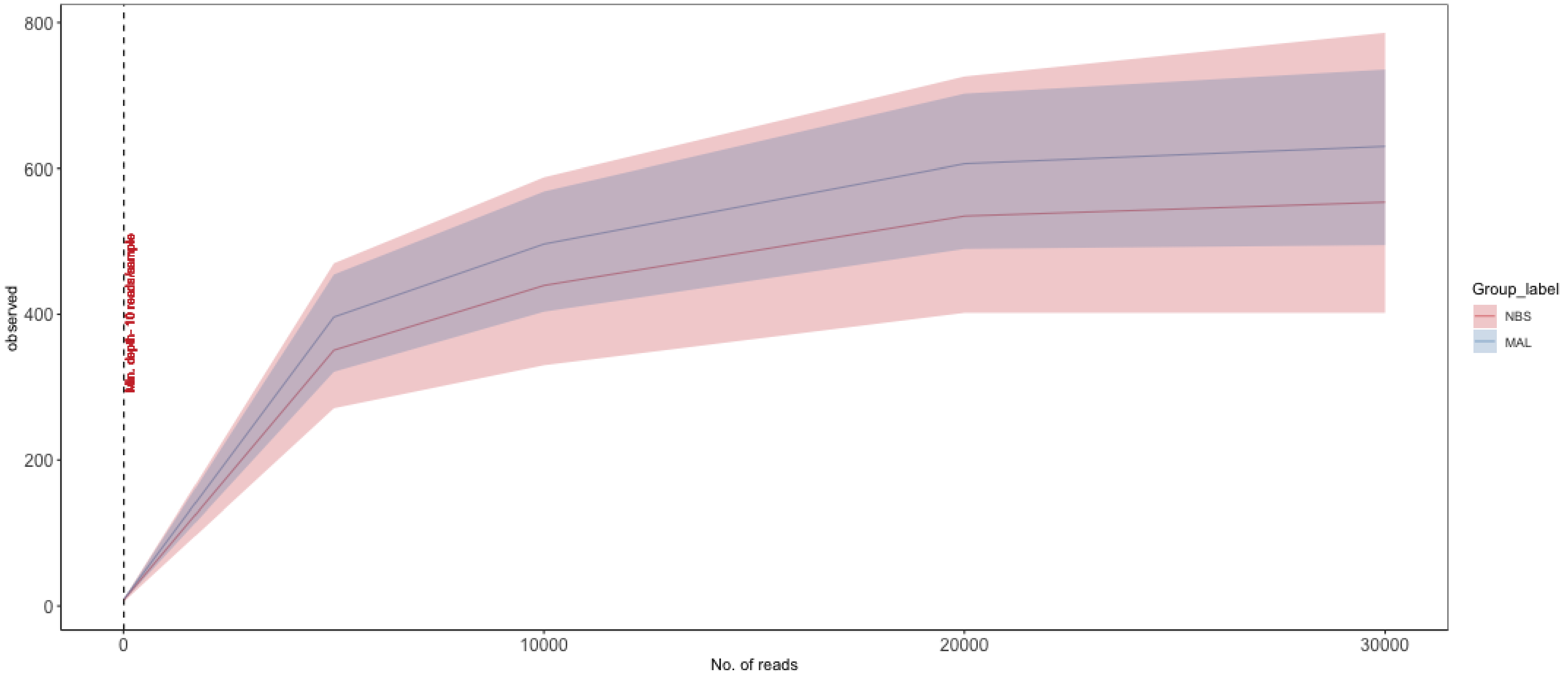


Rarefaction

Rarefaction is a technique to assess species richness from the results of sampling - mainly used in ecology. This curve is a plot of the number of species as a function of the number of samples. Rarefaction curves generally grow rapidly at first, as the most common species are found, but the curves plateau as only the rarest species remain to be sampled. We use this plot to see if we have reached an adequate level of sequencing depth for our samples.

```r
# set seed
set.seed(1)
# set subsample
subsamples = c(10, 5000, 10000, 20000, 30000)

rarecurve <- plot_alpha_rcurve(ps_M, index="observed",
                       subsamples = subsamples,
                       lower.conf = 0.025,
                       upper.conf = 0.975,
                       group="Group_label",
                       label.color = "brown3",
                       label.size = 3,
                       label.min = TRUE)
```

```
Warning in vegan::rrarefy(t(abundances(ps)), sample.size): some row sums < 'sample' and ar
rarefied

Warning in vegan::rrarefy(t(abundances(ps)), sample.size): some row sums < 'sample' and ar
```


800
600
400
200
0
observed
Min. depth- 10 reads/sample
0
10000
20000
30000
No. of reads
Group_label
NBS
MAL

Alpha diversity

Alpha diversity is the mean species diversity within a sample. There are different measurements/indexes. The most simplest being how many ASV/OTUs in each sample. Other common used measurements - chao1, shannon, inverse simpson,

Make using alpha diversity plots with statistical values using microbiomeutilities.

Produce alpha diversity plots using 4 measures - observed (i.e. number of OTUs), chao1, shannon and inverse simpson.

Statistical analysis with wilcoxon pair-wise test

```r
mycols = c("brown3", "steelblue")
obs_alpha_plot <- plot_diversity_stats(ps_M, group = "Group_label",
                            index = "observed",
                            label.format="p.format",
                            group.colors = mycols,
                            stats = TRUE)
```

Observed richness

Other forms of richness

Diversity

Evenness

Dominance

Rarity

Save your figures directly from R for bonus points on quality data reproducibility! This line with save your combined alpha diversity plots into a direcoty called *plots/*

```
ggsave("alphadiv_withpvalues.pdf", plot = alphadiv_wp,
path = "plots", width = 30, height = 30, units = "cm")
```

Distribution plot

This plot is good to give you an idea of the how taxa are distribution within the data. It will give you an idea about general trends in the data and help guide how further analysis.

```
# Bariatric Surgery
NBS_ps <- subset_samples(ps_M, Group_label=="NBS")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Taxa summary

Create a bar plot of phyla - showing difference in two groups `No bariatric surgery` vs `Malabsorptive`. Note that depending on your study present a barplot might a quick way to see patterns in your data generally they are not used to represent the community composition in your final figures.

Use these for visualizing at higher taxonomic levels (mostly phylum level). Remember also that you need to be careful when looking at relative microbiome abundance!

```r
mycols <- c("brown3", "steelblue")
grp_abund <- get_group_abundances(ps_M,
                                  level = "Phylum",
                                  group="Group",
                                  transform = "compositional")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Create a bar plot of order - showing difference in two groups `No bariatric surgery` vs `Malabsorptive`

```r
mycols <- c("brown3", "steelblue")
grp_abund <- get_group_abundances(ps_M,
                                  level = "Order",
                                  group="Group",
                                  transform = "compositional")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Composition barplot

To quickly visualize comparison in taxa between make a relative abundance barplot of taxa between sample group (aggregate taxa at family level).

```
# Get relative abundance and remove low abundant taxa
ps1.rel <- microbiome::transform(ps_M, "compositional")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Top taxa

Now we'll just take the top 5 family taxa. Lets plot the abundance between the two groups. Make some comments on the value of this type of analysis and how you might interpret the data (tell me also about the type of bacteria that were identified as well).

```
mycols <- c("brown3", "steelblue")
top_tax <- plot_taxa_boxplot(ps_M,
                        taxonomic.level = "Family",
                        top.otu = 6,
                        group = "Group_label",
                        add.violin= TRUE,
                        group.colors = mycols,
                        title = "Top six family",
                        keep.other = FALSE,
                        dot.size = 1)
```

```
For plotting purpuses the phy_tree will be removed
```

Heatmap

Rather than a barplot heatmaps are much better at presenting the microbiome composition in samples. These are commonly used in publications!

Create heatmap of core microbiome tutorial

Keep only taxa with count above zero and transform to compositional (relative abundance).

```
ps.prune <- prune_taxa(taxa_sums(ps_M) > 0, ps_M)
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

```
pseq.rel <- microbiome::transform(ps.prune, "compositional")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'
Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Aggregate data to genus level and make heat map of the most prevalent taxa.

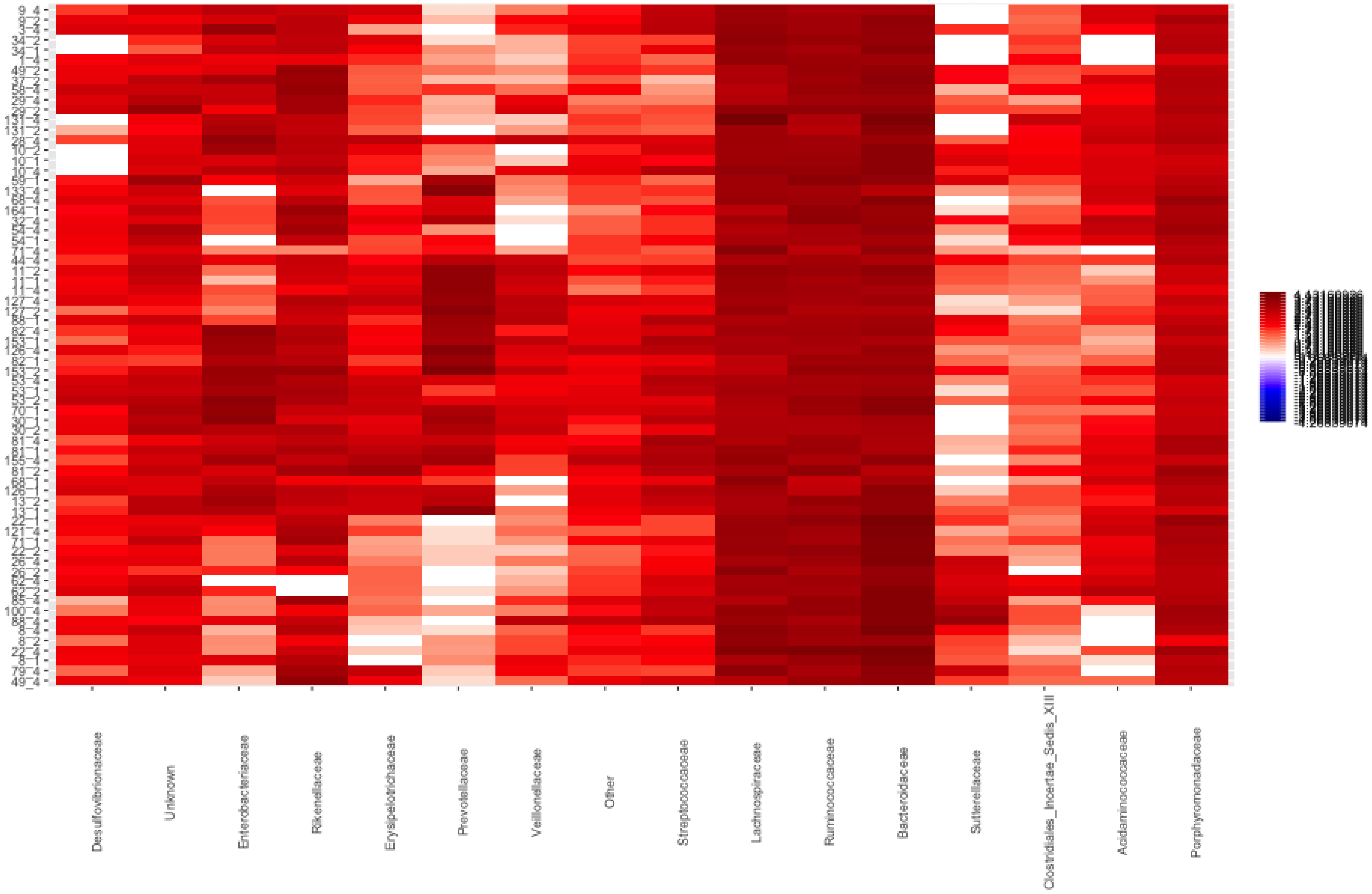
```r
library(RColorBrewer)

ps.m3.rel.gen <- aggregate_taxa(pseq.rel, "Genus")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Make a heat map of all samples - this can get a bit messy when you have a lot of samples but helpful to quickly see how different samples compare.

```r
ps1.rel <-aggregate_rare(ps_M, level = "Family", detection = 10, prevalence = 0.5)
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```


9_4
9_2
3_4
34_2
34_1
1_4
49_2
37_2
58_4
29_4
29_2
131_4
131_2
28_4
10_2
10_1
10_4
59_1
133_4
68_4
164_1
32_4
54_4
54_1
71_4
44_4
11_2
11_1
11_4
127_4
127_2
88_1
82_4
153_1
126_4
82_1
153_2
53_4
53_1
53_2
70_1
30_1
30_2
81_4
81_1
155_4
81_2
68_1
126_1
13_2
13_1
22_1
121_4
71_1
22_2
26_4
26_2
62_4
62_2
85_4
100_4
88_4
8_4
8_2
22_4
8_1
79_4
49_4
Desulfovibrionaceae
Unknown
Enterobacteriaceae
Rikenellaceae
Erysipelotrichaceae
Prevotellaceae
Veillonellaceae
Other
Streptococcaceae
Lachnospiraceae
Ruminococcaceae
Bacteroidaceae
Sutterellaceae
Clostridiales_Incertae_Sedis_XIII
Acidaminococcaceae
Porphyromonadaceae

Betadiversity

Variation of microbial communities between samples. Beta diversity shows the different between microbial communities from different environments.

Distance measures

Bray–Curtis dissimilarity

- based on abundance or read count data
- differences in microbial abundances between two samples (e.g., at species level) values are from 0 to 1 0 means both samples share the same species at exact the same abundances 1 means both samples have complete different species abundances

Jaccard distance

- based on presence or absence of species (does not include abundance information)
- different in microbial composition between two samples 0 means both samples share exact the same species 1 means both samples have no species in common

UniFrac

- sequence distances (phylogenetic tree)
- based on the fraction of branch length that is shared between two samples or unique to one or the other sample unweighted UniFrac: purely based on sequence distances (does not include abundance information) weighted UniFrac: branch lengths are weighted by relative abundances (includes both sequence and abundance information)

- Phyloseq
- MicrobiomeMiseq tutorial by Michelle Berr
- ampvis2

Ordination

Ordination methods are used to highlight differences between samples based on their microbial community composition - also referred to as distance- or (dis)similarity measures.

These techniques reduce the dimensionality of microbiome data sets so that a summary of the beta diversity relationships can be visualized in 2D or 3D plots. The principal coordinates (axis) each explains a certain fraction of the variability (formally called inertia). This creates a visual representation of the microbial community compositional differences among samples. Observations based on ordination plots can be substantiated with statistical analyses that assess the clusters.

There are many options for ordination. Broadly they can be broken into:

1. Implicit and Unconstrained (exploratory)

- Principal Components Analysis (PCA) using Euclidean distance
- Correspondence Analysis (CA) using Pearson chi-squared
- Detrended Correspondence Analysis (DCA) using chi-square

2. Implicit and Constrained (explanatory)

- Redundancy Analysis (RDA) using Euclidean distance
- Canonical Correspondance Analysis (CCA) using Pearson chi-squared

3. Explicit and Unconstrained (exploratory)

- Principal Coordinates Analysis (PCoA)
- non-metric Multidimensional Scaling (nMDS)
- Choose your own distance measure
  - Bray-Curtis - takes into account abundance (in this case abundance is the number of reads).
  - Pearson chi-squares - statistical test on randomness of differences
  - Jaccard - presence/absence
  - Chord
  - UniFrac, which incorporates phylogeny.
  - *Note*: if you set the distance metric to Euclidean then PCoA becomes Principal Components Analysis.

Some extra explanatory notes on PCoA and nMDS

PCoA is very similar to PCA, RDA, CA, and CCA in that they are all based on eigenan alysis: each of the resulting axes is an eigen vector associated with an eigen value, and all axes are orthogonal to each other. This means that all axes reveal unique information about the inertia in the data, and exactly how much inertia is indicated by the eigenvalue. When plotting the ordination result in an x/y scatterplot, the axis with the largest eigenvalue is plotted on the first axis, and the one with the second largest on the second axis.

Some extra explanatory notes on PCoA and nMDS

NMDS attempts to represent the pairwise dissimilarity between objects in a low-dimensional space. Can use any dissimilarity coefficient or distance measure. NMDS is a rank-based approach based on an iterative algorithm. While information about the magnitude of distances is lost, rank-based methods are generally more robust to data which do not have an identifiable distribution. NMDS routines often begin by random placement of data objects in ordination space. The algorithm then begins to refine this placement by an iterative process, attempting to find an ordination in which ordinated object distances closely match the order of object dissimilarities in the original distance matrix. The stress value reflects how well the ordination summarizes the observed distances among the samples.

Detrended correspondence analysis (DCA)

`Implicit and Unconstrained (exploratory)`

Ordination of samples using DCA. Leave distance blank, so default is chi-square.

```r
# Ordinate the data
set.seed(4235421)
mycols <- c("brown3", "steelblue")
# proj <- get_ordination(pseq, "MDS", "bray")
ord.dca <- ordinate(ps_M, "DCA")
ord_DCA = plot_ordination(ps_M, ord.dca, color = "Group_label") +
                geom_point(size = 5) + scale_color_manual(values=mycols) + stat_ellipse() +
```

Canonical correspondence analysis (CCA)

`Implicit and Constrained (explanatory)`

Ordination of samples using CCA methods using Pearson chi-squared. Constrained variable used as `Group_label`.

```r
mycols <- c("brown3", "steelblue")
pseq.cca <- ordinate(ps_M, "CCA", cca = "Group_label")
ord_CCA <- plot_ordination(ps_M, pseq.cca, color = "Group_label")
ord_CCA <- ord_CCA + geom_point(size = 4) +
  scale_color_manual(values=mycols) + stat_ellipse() + theme_biome_utils()
```

Redundancy analysis (RDA)

`Implicit and Constrained (explanatory)`

Ordination of samples using RDA methods using Euclidean distance. Constrained variable used as `Group_label`.

```r
mycols <- c("brown3", "steelblue")
pseq.rda <- ordinate(ps_M, "RDA", cca = "Group_label")
ord_RDA <- plot_ordination(ps_M, pseq.rda, color = "Group_label")
ord_RDA <- ord_RDA + geom_point(size = 4) +
  scale_color_manual(values=mycols) + stat_ellipse() + theme_biome_utils()
```

Principal Coordinates Analysis (PCoA)

PCoA is very similar to PCA, RDA, CA, and CCA in that they are all based on eigenanalysis: each of the resulting axes is an eigenvector associated with an eigenvalue, and all axes are orthogonal to each other. This means that all axes reveal unique information about the inertia in the data, and exactly how much inertia is indicated by the eigenvalue.

Ordination of samples using PCoA methods and **jaccard** (presence/absence) distance measure

```r
# Ordinate the data
set.seed(4235421)
mycols <- c("brown3", "steelblue")
# proj <- get_ordination(pseq, "MDS", "bray")
ord.pcoa.jac <- ordinate(ps_M, "PCoA", "jaccard")
ord_PCoA_jac = plot_ordination(ps_M, ord.pcoa.jac, color = "Group_label") +
                geom_point(size = 5) + scale_color_manual(values=mycols) + stat_ellipse()
```

Ordination of samples using PCoA methods and **bray curtis** (abundance) distance measure.

```r
# Ordinate the data
set.seed(4235421)
mycols <- c("brown3", "steelblue")
ord.pcoa.bray <- ordinate(ps_M, "PCoA", "bray")
ord_PCoA_bray = plot_ordination(ps_M, ord.pcoa.bray, color = "Group_label") +
                geom_point(size = 5) + scale_color_manual(values=mycols) + stat_ellipse() +
```

Principal Coordinates Analysis (PCoA) with unifrac

Unifrac analysis takes into account not only the differences in OTUs/ASVs but also takes into account the phylogeny of the taxa. I.e. how closely related are the taxa.

We can perform unweighted (using presence/absence abundance like jaccard) or weighted (incorporating abundance data - like bray curtis).

Unweighted unifrac

```r
# Ordinate the data
set.seed(4235421)
mycols <- c("brown3", "steelblue")
ord_pcoa_ufuw <- ordinate(ps_M, "PCoA", "unifrac", weighted=FALSE)
```

```
Warning in UniFrac(physeq, ...): Randomly assigning root as --
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGAACTCCTATGAACGAGGTTTCGGCCAAGTGAATAGGATGTTTAGTGGCGGACGGG
-- in the phylogenetic tree in the data you provided.

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'
```

Weighted unifrac

```r
mycols <- c("brown3", "steelblue")
ord_pcoa_ufw = ordinate(ps_M, "PCoA", "unifrac", weighted=TRUE)
```

```
Warning in UniFrac(physeq, ...): Randomly assigning root as --
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAAGCACTTATCTTTGATTCTTCGGATGAAGAGATTTGTGACTGAGTGGCGGA
-- in the phylogenetic tree in the data you provided.

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'
```

Non-metric Multidimensional Scaling (nMDS)

Finally lets perform ordination using Non-metric Multidimensional Scaling. We'll use the unifrac distance measure which takes into account phylogeny and also the `WEIGHTED` option.

```r
# Ordinate the data
set.seed(4235421)
mycols <- c("brown3", "steelblue")
ord_nmds_ufw <- ordinate(ps_M, "NMDS", "unifrac", weighted=TRUE)
```

```
Warning in UniFrac(physeq, ...): Randomly assigning root as --
GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGAACTCCTATGAACGAGGTTTCGGCCAAGTGAATAGGATGTTTAGTGGCGGACGGG
-- in the phylogenetic tree in the data you provided.

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Statistical analysis

Here we'll perform a statistical analysis on beta diversity.

See tutorial here.

Differences by `Group_label` using ANOVA

```
# Transform data to hellinger
pseq.rel <- microbiome::transform(ps_M, "hellinger")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'
```

Hierarchical cluster analysis

Beta diversity metrics can assess the differences between microbial communities. It can be visualized with PCA or PCoA, this can also be visualized with hierarchical clustering.

Function from MicrobiotaProcess using analysis based on ggtree.

```r
## All samples - detailed, include species and SampleCategory
clust_all <- get_clust(obj=ps_M, distmethod="euclidean",
                       method="hellinger", hclustmethod="average")
```

```
Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'

Also defined by 'tidytree'

Found more than one class "phylo" in cache; using the first, from namespace 'phyloseq'
```

EXPLORE

This workshop was intended to give you a flavour of what microbiome analysis can involve.

You are encouraged to explore this topic and expand on the analysis we have done here.

The internet is a wealth of options.

Here is one of my favourite links for all things microbiome and R.
R environment based tools for microbiome data

Thanks!

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siobhonlegan.com/BIO514-microbiome

siobhon-egan.github.io/BIO514-microbiome

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