

General Information  
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What is microbiome?  
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Technology  
oooooooooooo

Ecological analysis  
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# Statistical methods for microbiome data

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Johns Hopkins University

3rd Term, 2020-2021

## General Information

- ▶ Classroom: N. Wolfe building. W3031
  - ▶ Lecture: Friday 10:30AM -11:50AM (EST)
  - ▶ Instructor: Ni Zhao (nzhao10@jhu.edu)
  - ▶ Assessment: critique on a recent preprint paper

## In-person environment

- ▶ Face covering are required for all students & faculties during in-door environment
  - ▶ *If you feel sick, please stay at home and get tested*
    - ▶ Even if you feel it is just a “common cold”.
    - ▶ Zoom recording will be provided.
  - ▶ In rare cases, virtual classes may be scheduled. Pay attention to class emails.

# Course content

## Chapter 1. Introduction

- ▶ sequencing procedures
- ▶ data structure

## Chapter 2. Ecological Methods

- ▶ alpha diversities, beta diversities.
- ▶ PERMANOVA, MiRKAT

## Chapter 3. Compositional models

- ▶ Aitchison distance
- ▶ Log-contrast models

## Chapter 4. Bias in microbiome data and how to address it.

## More about the course

- ▶ Material available at my github site?

General Information



What is microbiome?



Technology



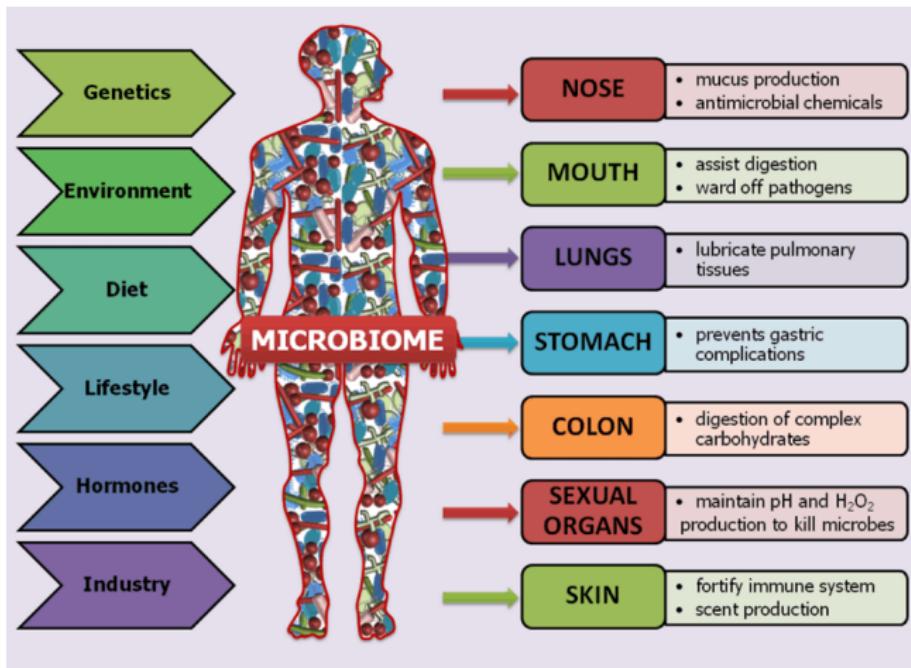
Ecological analysis



# Chapter 1: Introduction

# What is microbiome?

The collection of all microorganisms that co-exist with human being.



## The Importance of the **MICROBIOME**

### By the Numbers

**10-100 trillion**

Number of symbiotic microbial cells harbored by each person, primarily bacteria in the gut, that make up the human microbiota

**>10,000**

Number of different microbe species researchers have identified living in the human body

**100 to 1**

The genes in our microbiome outnumber the genes in our genome by about 100 to 1

**22,000**

Approximate number genes in the human gene catalog

**99.9%**

Percentage individual humans are identical to one another in terms of host genome

**90%**

Up to 90% of all disease can be traced in some way back to the gut and health of microbiome

**10X**

There are 10 times as many outside organisms as there are human cells in the human body

**3.3 million**

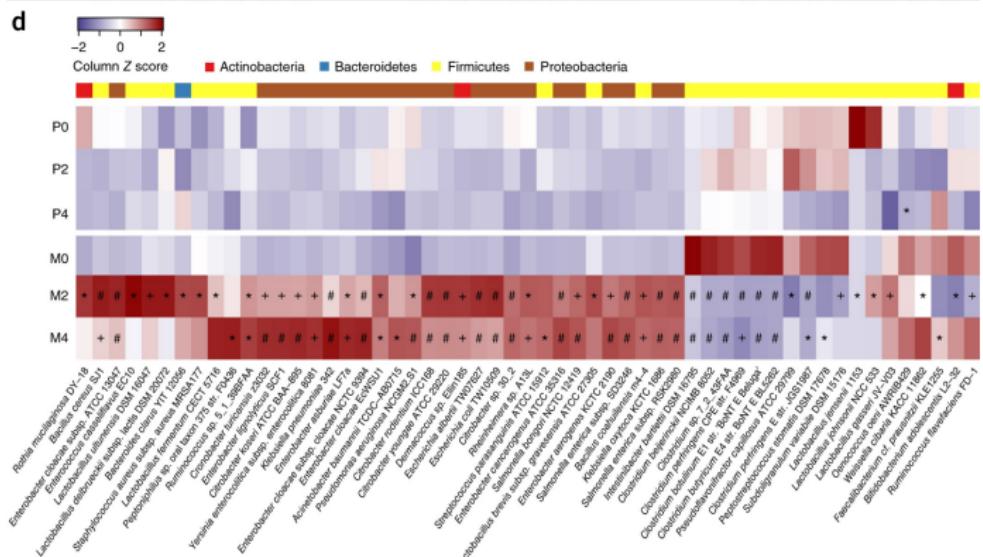
Number of non-redundant genes in the human gut microbiome

**80%- 90%**

Percentage individual humans are different from another in terms of the microbiome

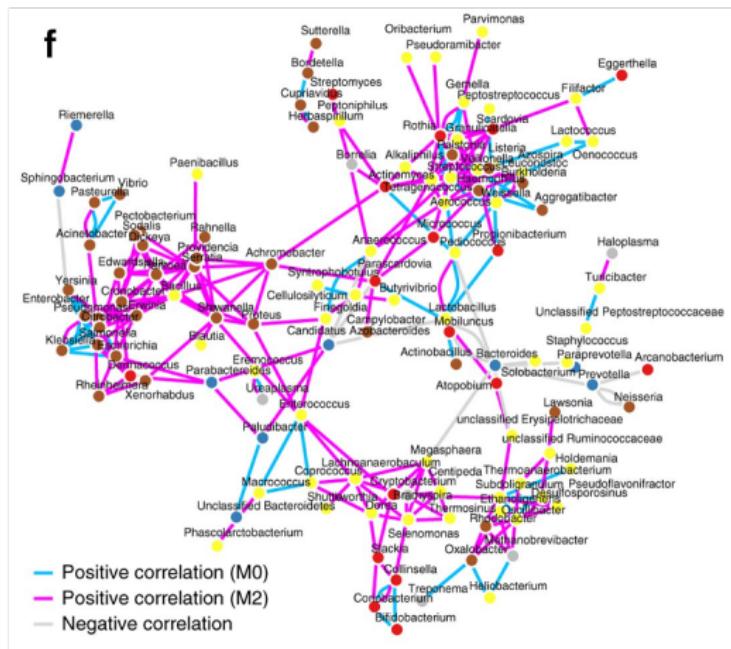
## Example 1: Diabetes and Metformin treatment

Metformin treatment promotes rapid changes in the composition of the gut microbiota, contributing to the therapeutic effects of the drug.



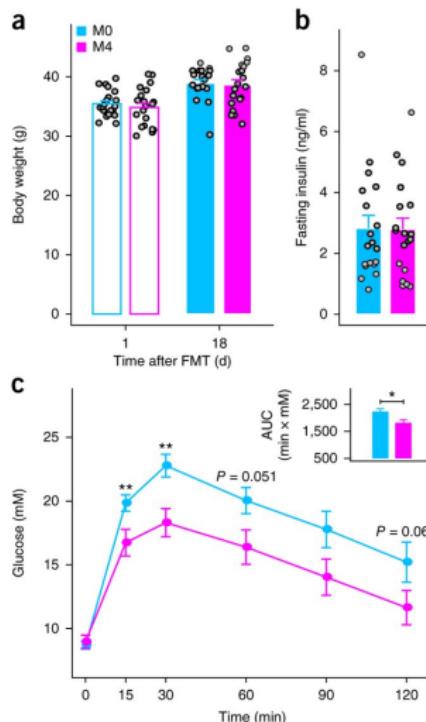
## Example 1: Diabetes and Metformin treatment

Metformin treatment promotes rapid changes in the structure of the gut microbiota.

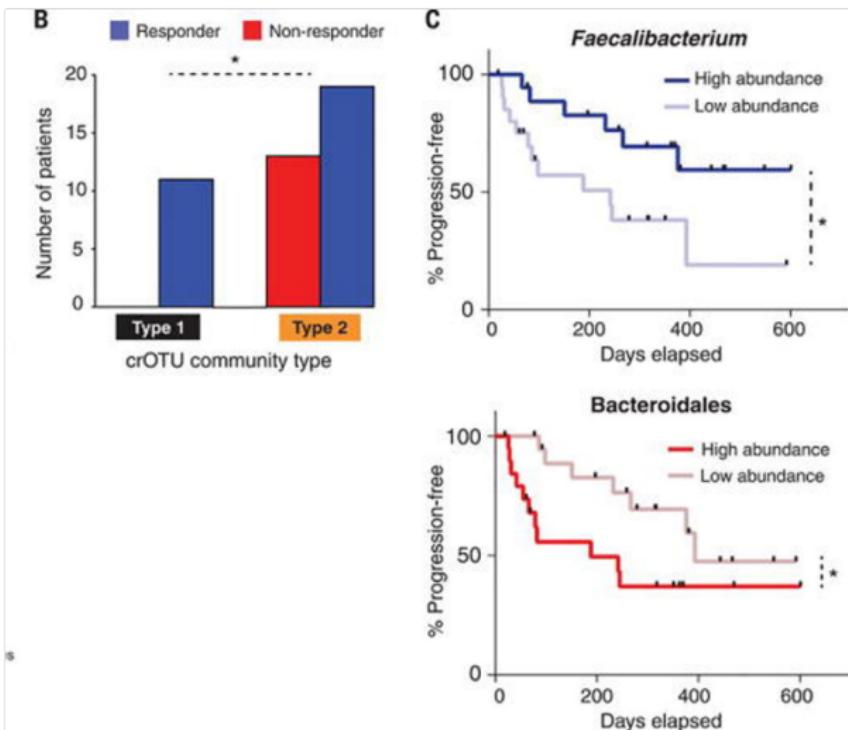


## Example 1: Diabetes and Metformin treatment

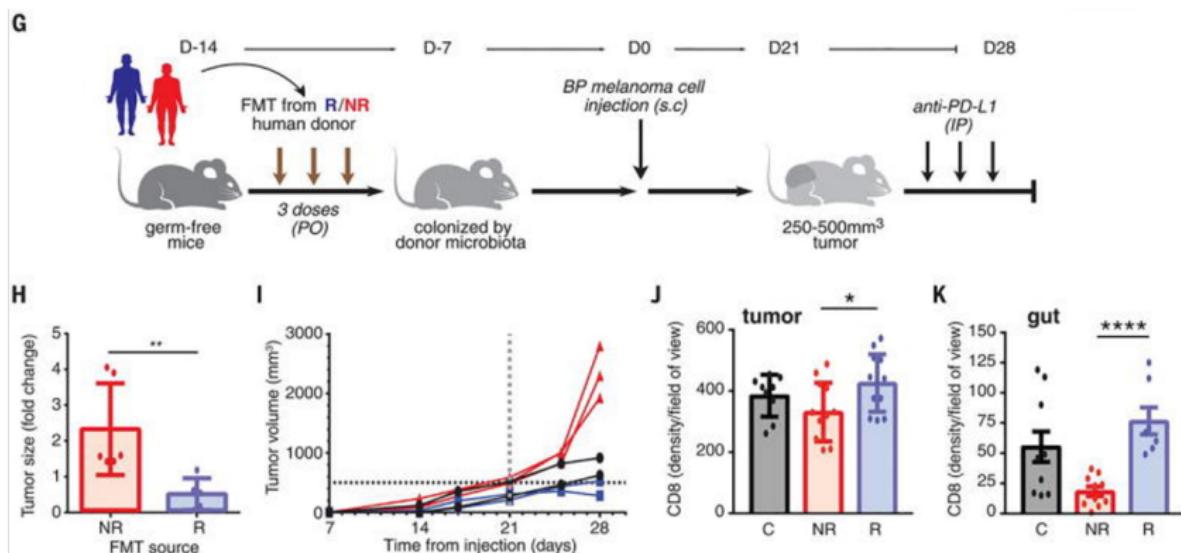
Metformin-altered microbiota improves glucose tolerance.

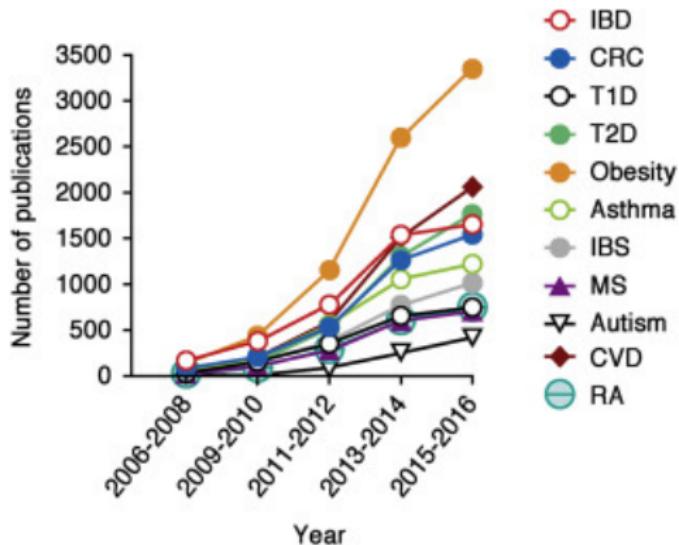


## Example 2: Immunotherapy and gut microbiome

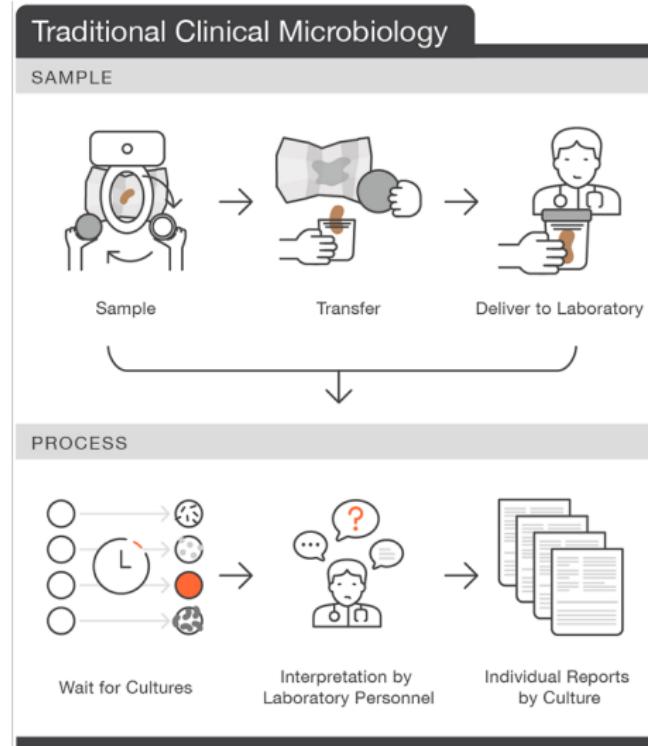


## Example 2: Immunotherapy and gut microbiome

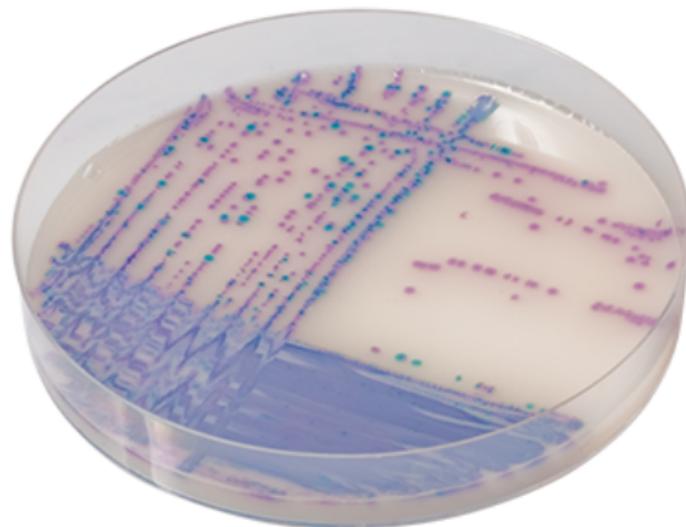
Gopalakrishnan et al., *Science*, 2018

Butto et al., *Journal of Allergy and Clinical Immunology*, 2017

# Traditional Microbiology

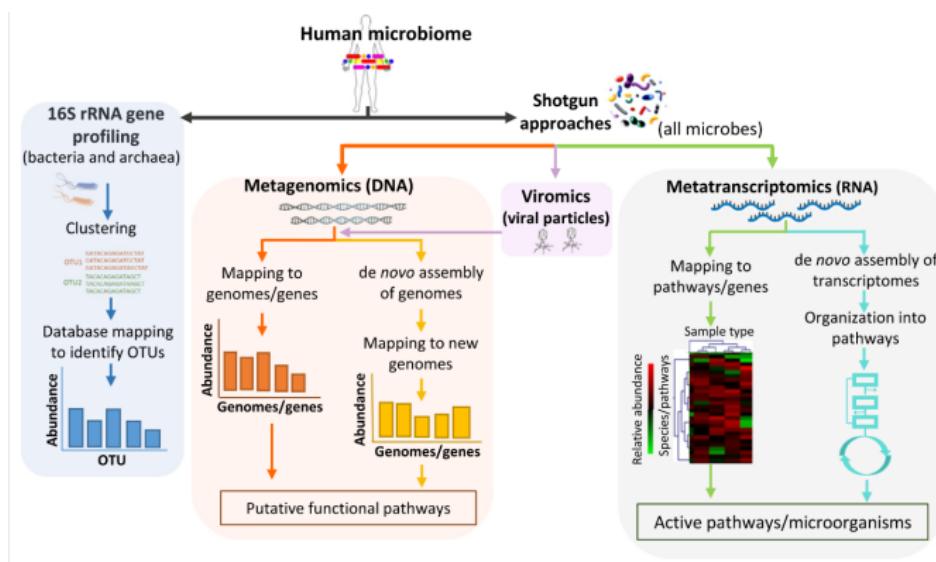


## Cell Culture



<https://asm.org/Articles/2020/September/How-CHROMagar-TM-Revolutionized-Bacterial-Identifi>

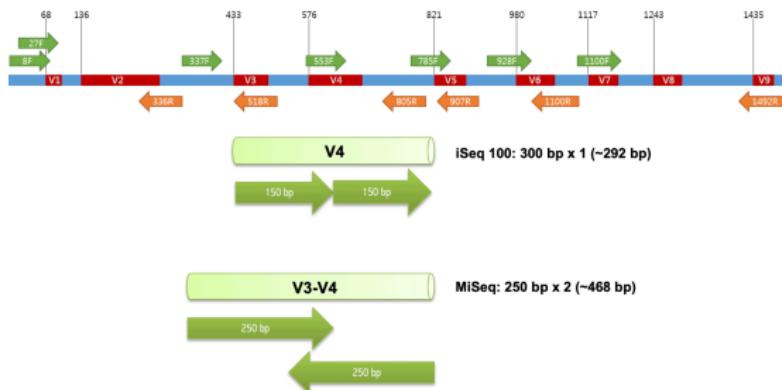
## Next-generation sequencing for microbiome assessment



Bikel et al., *Computational and Structural Biotechnology Journal*, 2015

# Marker gene sequencing, a.k.a., amplicon sequencing

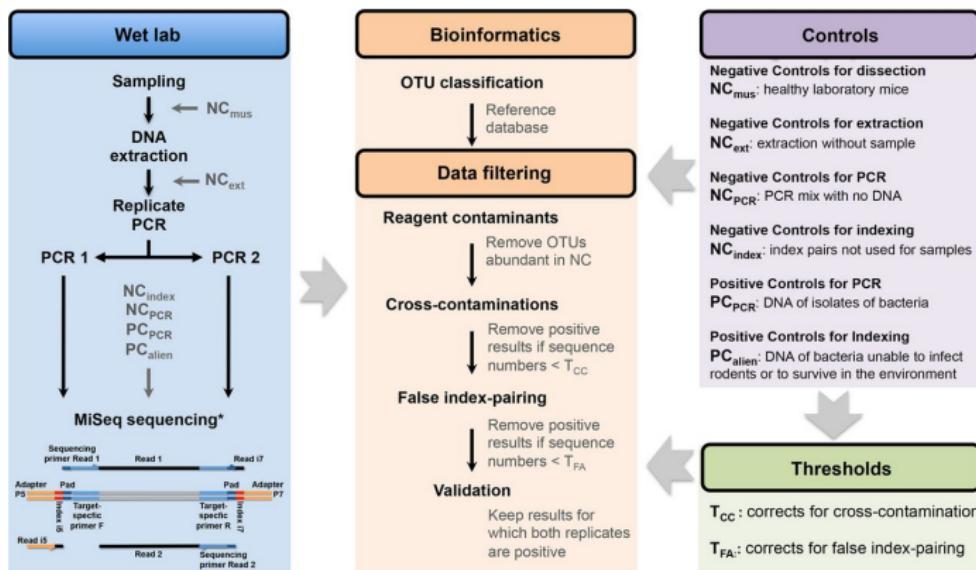
Most commonly used marker gene: 16s rRNA gene



<https://help.ezbiocloud.net/16s-rrna-and-16s-rrna-gene/>

Other marker genes include: 18S rRNA, ITS (internal transcribed spacer) for fungi identification

# Marker gene sequencing, a.k.a., amplicon sequencing



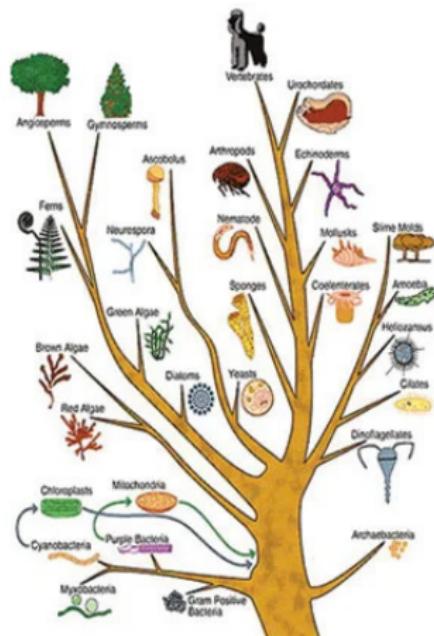
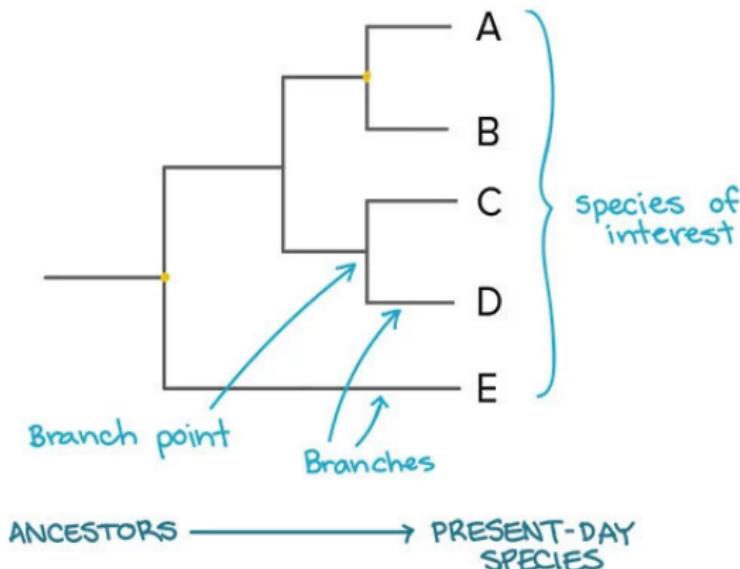
## OTUs vs ASVs

- ▶ Operational Taxonomic Units (OTUs): clustering based approach
  - ▶ Usually, 97% similarity for genus level.
  - ▶ Possible to match to existing database: SALIVA, greengene etc.
  - ▶ de novo OTUs defined in two different data sets cannot be compared.
- ▶ Amplicon sequence variants (ASVs): *Callahan, et al., Nature Methods, 2016*
  - ▶ Non-clustering based approach
  - ▶ ASVs defined in two different data sets cannot be compared.
  - ▶ Possible to match to existing database: SALIVA, greengene etc.
- ▶ Both are provided in Qiime2 pipeline.

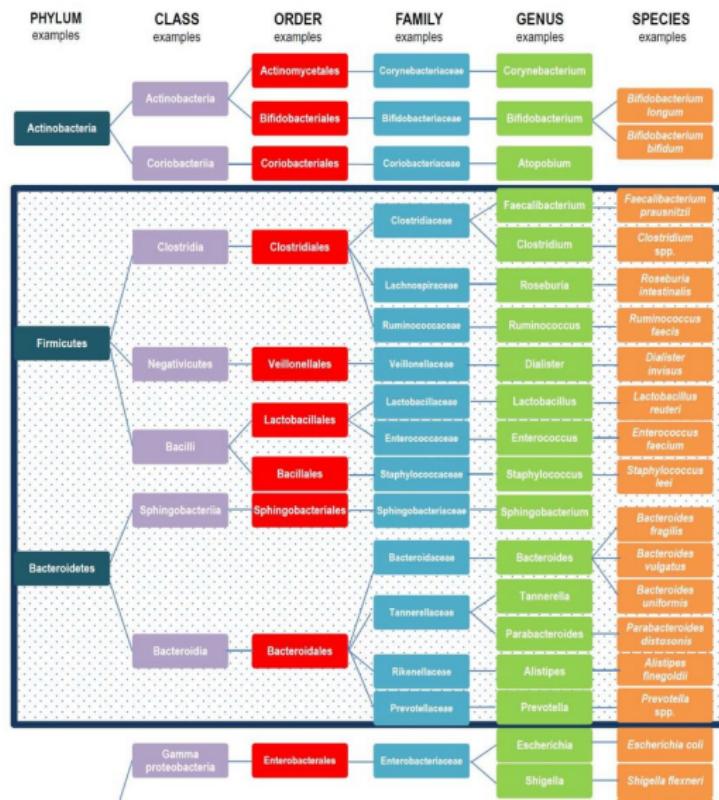
## Typical data from 16s sequencing

# Constructed from biom file							
#OTU ID	66058686	66165152	66167056	66158660	66167050	66167120	
4386761	1.0	1.0	1.0	0.0	0.0	0.0	0.0
1800048	0.0	0.0	1.0	3.0	1.0	2.0	0.0
358030	0.0	0.0	0.0	0.0	0.0	33.0	5.0
309284	0.0	0.0	0.0	0.0	0.0	0.0	1.0
4030157	0.0	0.0	0.0	0.0	0.0	1.0	0.0
296165	152.0	9.0	36.0	16.0	2.0	0.0	1.0
309873	0.0	0.0	0.0	0.0	0.0	0.0	0.0
337735	0.0	0.0	1.0	0.0	0.0	0.0	0.0
592160	0.0	0.0	0.0	0.0	0.0	0.0	0.0
189110	0.0	0.0	2.0	0.0	0.0	0.0	0.0
327049	0.0	0.0	0.0	0.0	0.0	0.0	0.0
193591	0.0	7.0	6.0	4.0	1.0	2.0	8.0
4355379	0.0	0.0	0.0	0.0	0.0	0.0	0.0
782984	0.0	0.0	0.0	0.0	0.0	1.0	0.0
330294	0.0	0.0	0.0	0.0	0.0	55.0	0.0
177224	0.0	0.0	0.0	0.0	1.0	0.0	0.0
177222	1.0	0.0	4.0	0.0	0.0	3.0	0.0
569210	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3589405	0.0	0.0	0.0	0.0	0.0	0.0	0.0
949863	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1026778	0.0	0.0	0.0	0.0	0.0	0.0	2.0

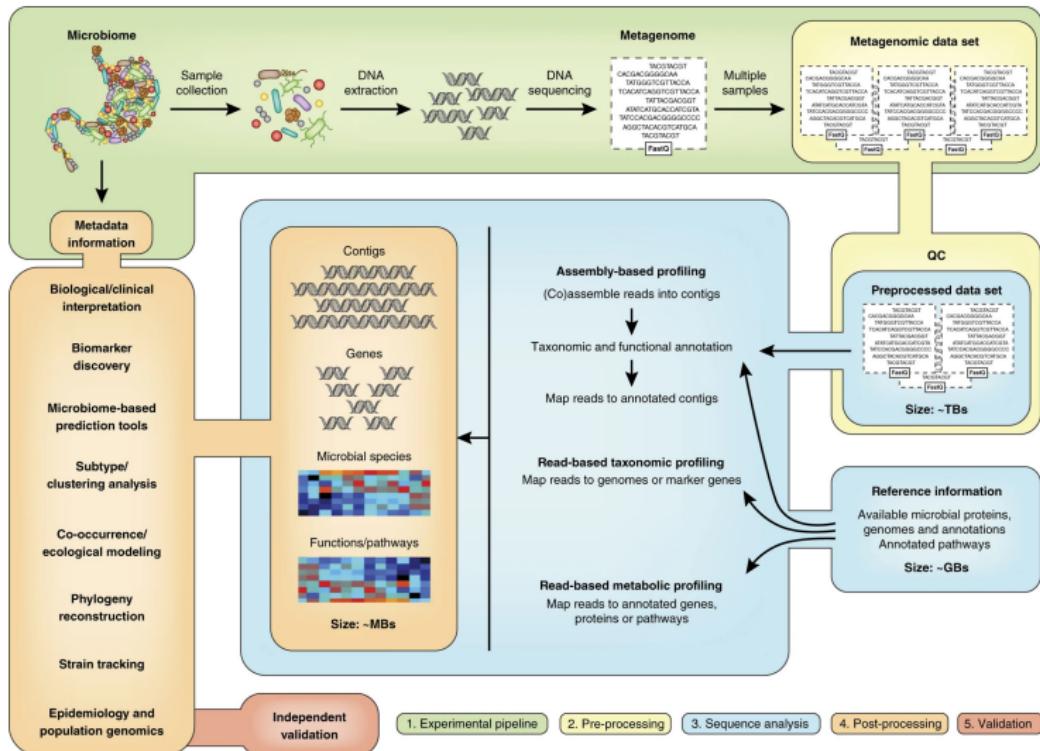
# Phylogenetic tree



# Taxonomy tree



# Shotgun metagenomics sequencing



## Compared to marker gene sequencing, shotgun sequencing

- ▶ sequences all genomic DNA in your sample.
- ▶ able to obtain species level resolution.
- ▶ obtain genomic and functional content in addition to taxa information.  
(inferring functional pathway is possible for marker gene sequencing:  
PiCRUSt)
  - ▶ Antibiotic-resistant genes.
  - ▶ Strain specific information.
- ▶ Other possible use: inference of bacteria growth rate.
- ▶ More expensive
- ▶ Higher false positives
- ▶ Host DNA interference

## Community structure

Healthy



Dysbiosis



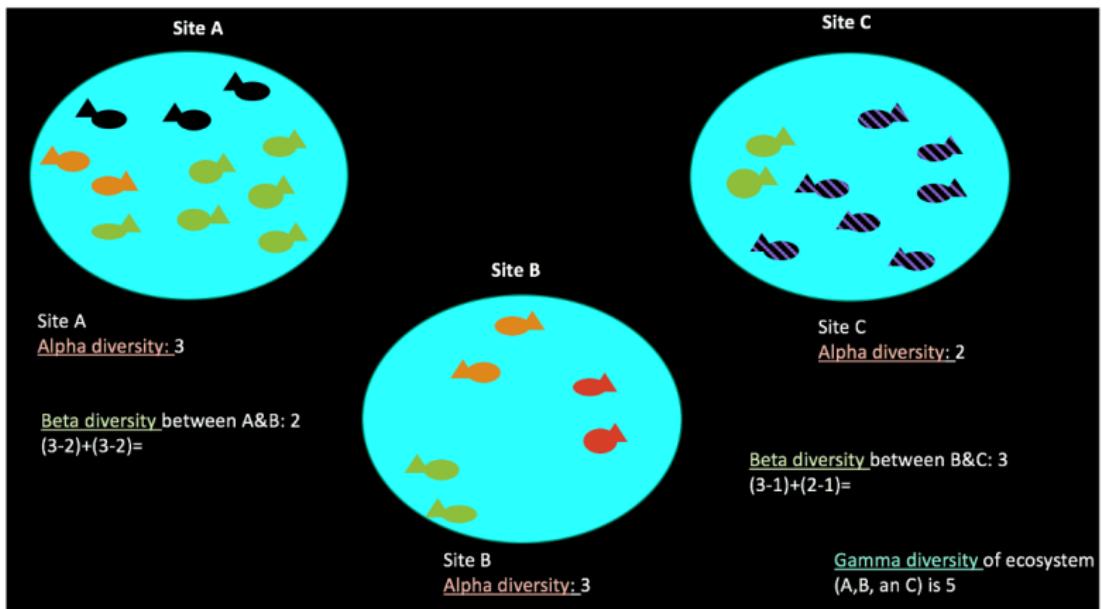
Gut



Vaginal



# Diversities

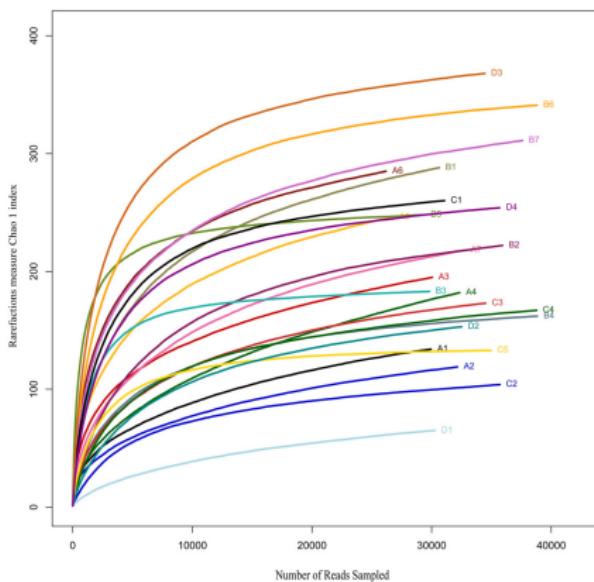


# Alpha diversity

Diversity	Description	Formula
Non-phylogenetically based		
Observed Species	No. of unique taxa in a sample	Number of species
Chao 1	Adding a correction to observed species	$S_{obs} + \frac{n_1^2}{2n_2}$
Shannon	Both richness and evenness	$-\sum_{i=1}^s p_i \log p_i$
Simpson reciprocal	Both richness and evenness	$\frac{N(N - 1)}{n(n - 1)}$
Pielou's evenness	evenness	$\frac{H}{H_{max}}$
Phylogenetically based		
PD-Tree	Based on where the bacteria are in the evolution tree	

# Diversity depends on the intensity of sampling

## ► Rarefaction



## Rarefying: controversial yet popular

- ▶ Appears in most standard analysis pipelines.
- ▶ A nonparametric robust approach that appears to work well in real data.
- ▶ McMurdie and Holmes, 2014
  - ▶ Rarefying throws away data.
  - ▶ Inadmissible in both clustering of communities and DA analysis.
- ▶ Weiss et al, 2017
  - ▶ “Rarefying more clearly clusters samples according to biological origin than other normalization techniques do for ordination metrics based on presence or absence.”

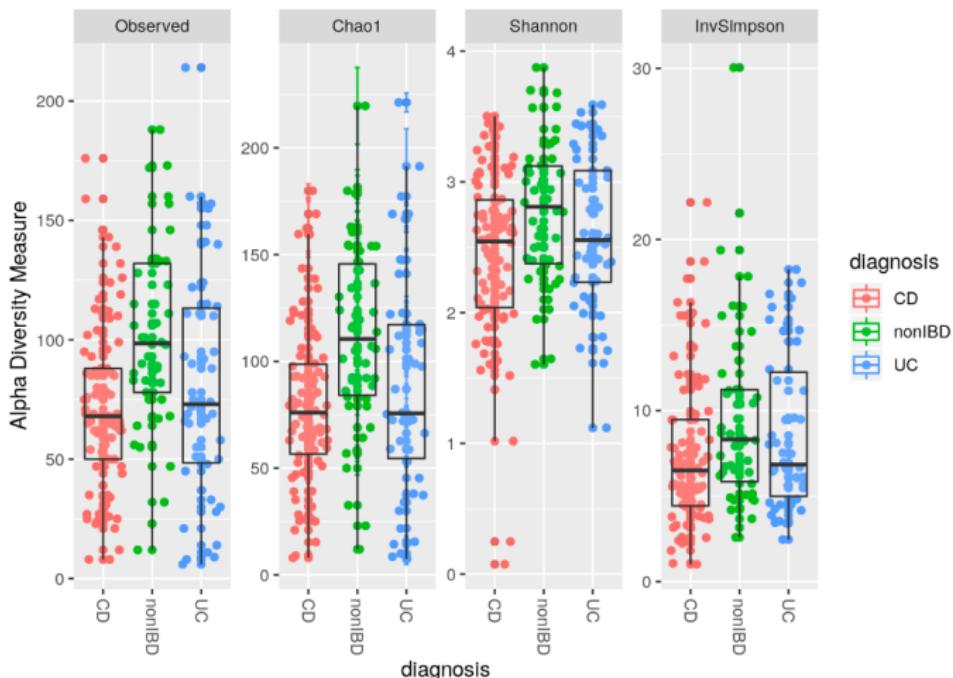
## Other approaches: estimate the unobserved taxa.

- ▶  $W_{ij}$  read counts in sample  $i$ , taxon  $j$ .
- ▶  $N_i$ : total number of (all, including nonobserved) taxa in sample  $i$ .
- ▶ Assumes a Poisson Multinomial distribution:  $N_i \sim \text{Pois}(\nu_i)$
- ▶  $f_{X_i}^*(W_{i1}, \dots, W_{ip} | N_i) = \frac{N_i!}{\prod_{j=1}^p W_{ij}!} \prod_{j=1}^p X_{ij}^{*W_{ij}}$
- ▶ MLE of  $\hat{X}_{ij} = W_{ij} / \sum_{k=1}^p W_{ik}$ , which is not very suitable due to dropout.
- ▶ Regularized approach provided.

Yuanpei Cao et al., *Biometrika*, 2020

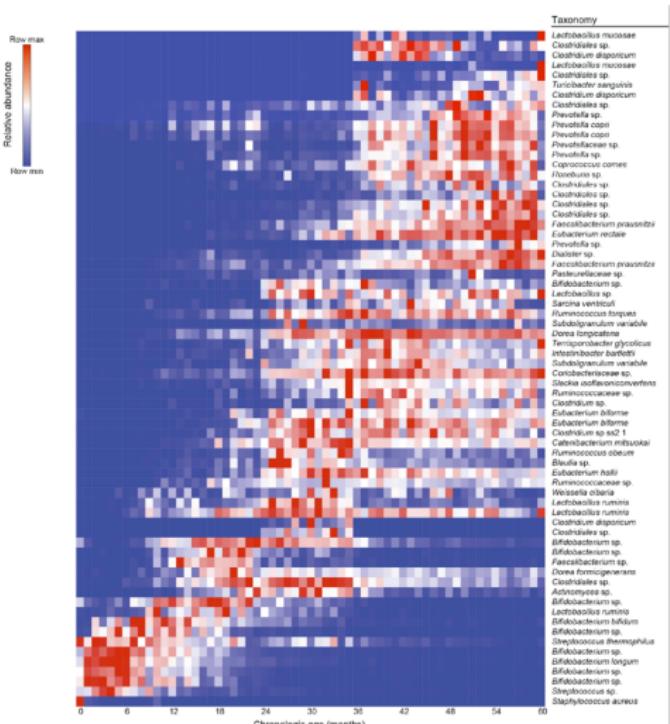
# Alpha diversity analysis

As alpha diversity is univariate, after it is calculated, apply your STATS 101.



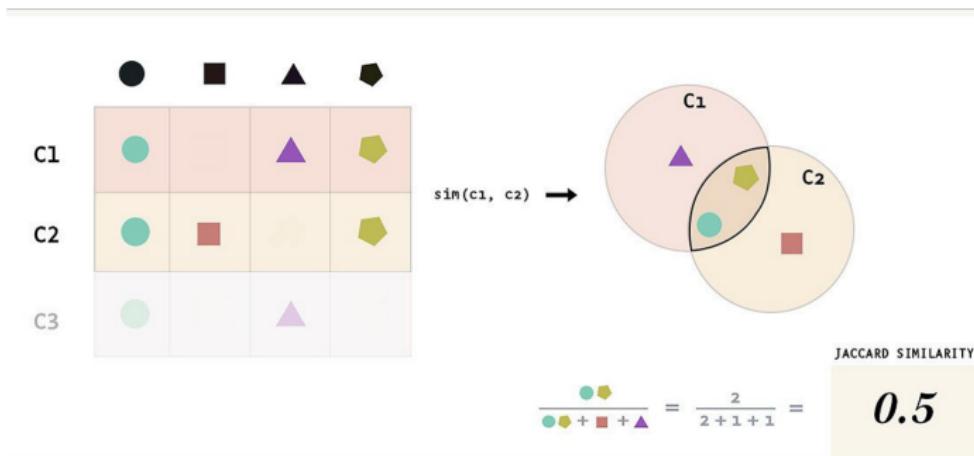
# Defining normal gut microbiota development

- ▶ Healthy members of Bangladeshi birth cohort
- ▶ Monthly sampling (1-60 months)
- ▶ 16S rDNA-based analysis
- ▶ AAAS2022, Jeff Gordon



Beta diversities: captures the dis-similarity between samples.

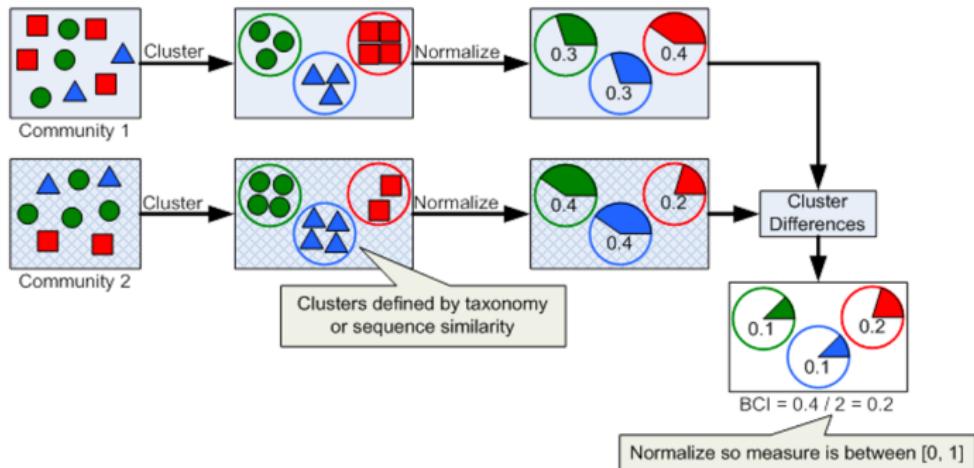
$$\text{Jaccard index: } J(A, B) = \frac{|A \cap B|}{|A \cup B|}$$



Jaccard distance = 1 - Jaccard index.

<https://www.learndatasci.com>

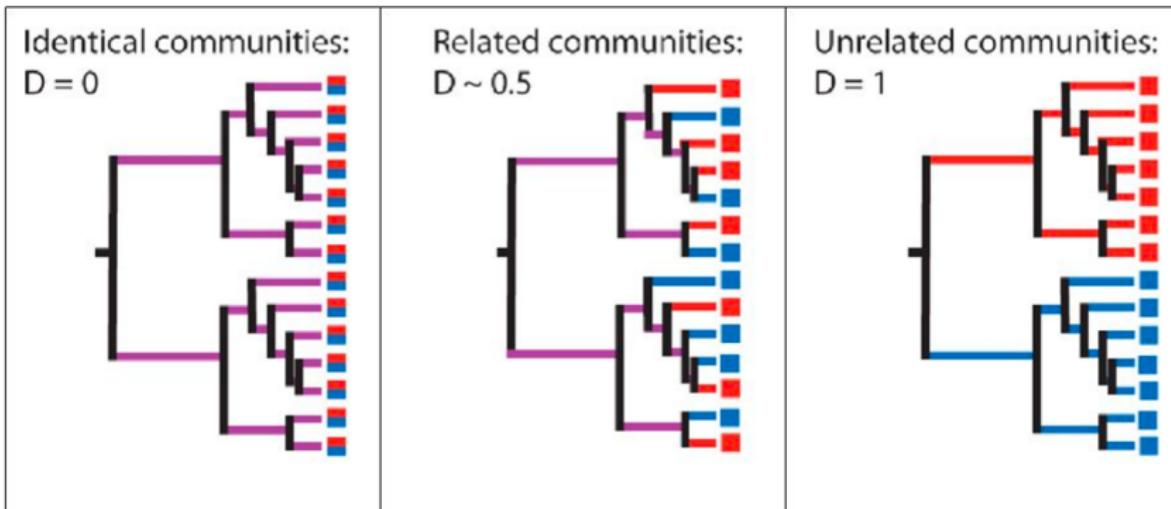
# Bray-Curtis Distance



$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j} = 1 - 2 \times \frac{0.3+0.3+0.2}{2} = 0.2$$

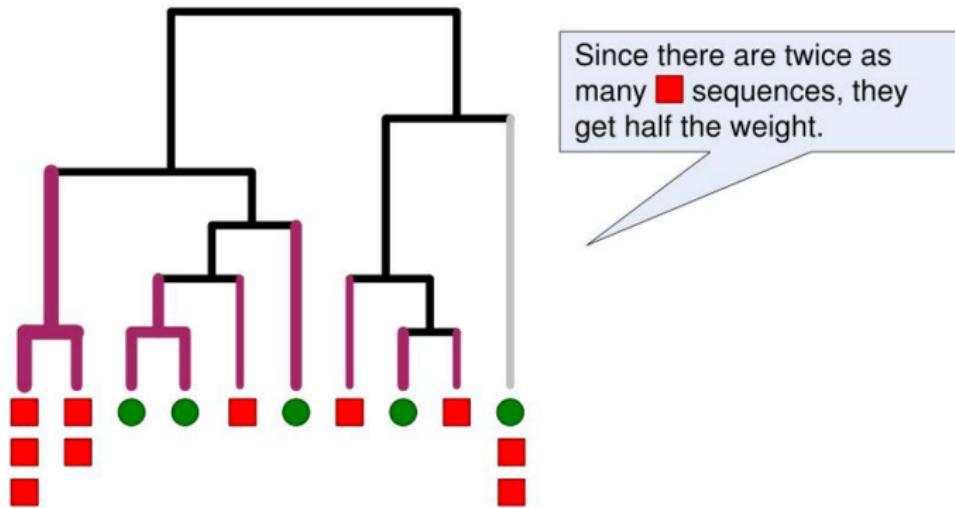
## Unique Fraction (UniFrac) metric

- Qualitative phylogenetic  $\beta$  diversity.
- Distance = fraction of the total branch length that is unique to any particular environment.



# Weighted UniFrac

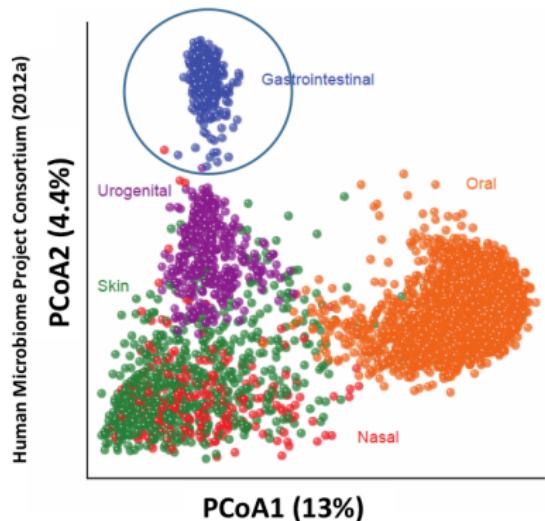
- What about the relative abundance of sequences (i.e., evenness)?



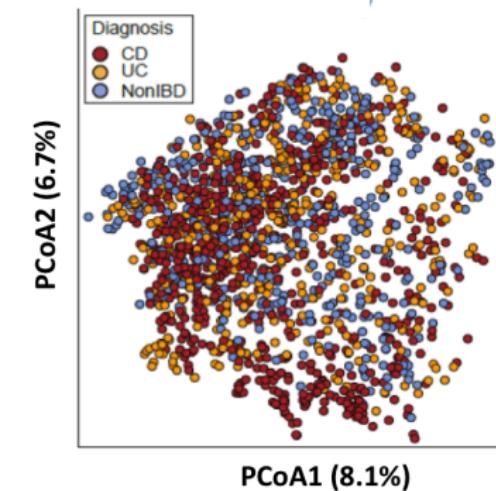
# Commonly used beta diversities

Diversity	Description	Formula
UniFrac	Qualitative, Phylogenetics-based	$\sum_{i=1}^n \frac{b_i  I(p_i^A > 0) - I(p_i^B > 0) }{\sum_{i=1}^n b_i}$
Weighted UniFrac	Quantitative, Phylogenetics-based	$\frac{\sum_{i=1}^n b_i  p_i^A - p_i^B }{\sum_{i=1}^n b_i (p_i^A + p_i^B)}$
Generalized UniFrac	Compromise between the previous two	$\frac{\sum_{i=1}^n b_i (p_i^A + p_i^B)^\alpha   \frac{p_i^A - p_i^B}{p_i^A + p_i^B}  }{\sum_{i=1}^n b_i (p_i^A + p_i^B)^\alpha}$
Bray-Curtis	Quantitative	$1 - \frac{2C_{ij}}{S_i + S_j}$
Jaccard	Qualitative	$1 - \frac{ A \cap B }{ A \cup B }$

# Principal Coordinate Plot



Microbial community composition in each body region is distinct.



But large-scale community composition alone cannot differentiate host phenotypes.

# PCoA

- ▶ Distance to similarity  $D = [D_{hi}]$ ,  $A = -\frac{1}{2}[D_{hi}^2]$
- ▶ Gower centering (1966).

$$G = \left(I - \frac{11'}{n}\right) A \left(I - \frac{11'}{n}\right)$$

- ▶ Eigen-decomposition of  $G$
- ▶ Project the distance metric into lower dimensional space

Note: metric multidimensional scaling vs nonmetric multidimensional scaling (MDS vs NMDS)

# PERMANOVA

*Austral Ecology* (2001) **26**, 32–46

## A new method for non-parametric multivariate analysis of variance

MARTI J. ANDERSON

*Centre for Research on Ecological Impacts of Coastal Cities, Marine Ecology Laboratory,  
University of Sydney, New South Wales 2006, Australia*

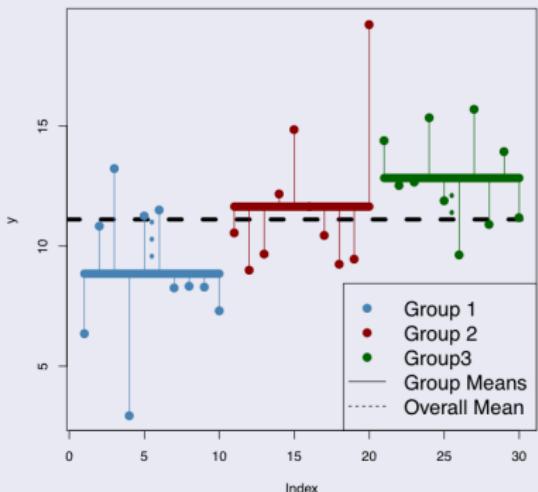


**Abstract** Hypothesis-testing methods for multivariate data are needed to make rigorous inferences about the effects of factors and their interactions in experiments. Analysis of variance

- ▶ Non-parametric approach combined with ecological distance measures!

## Question : How is univariate ANOVA calculated?

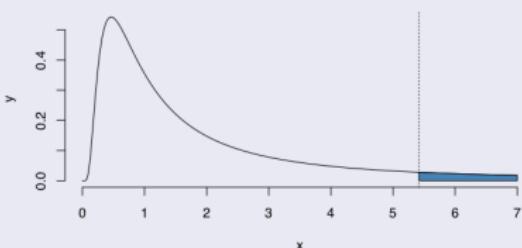
### From univariate...

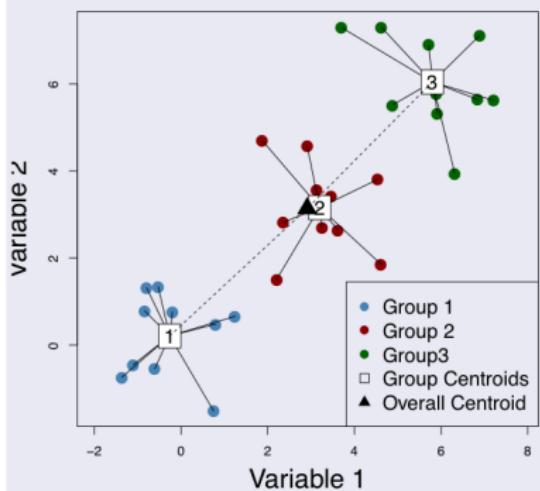


$$SS_{total} = SS_{residual} + SS_{group}$$

$$F - ratio = \frac{SS_{group}}{SS_{residual}} \cdot \frac{df_{residual}}{df_{group}}$$

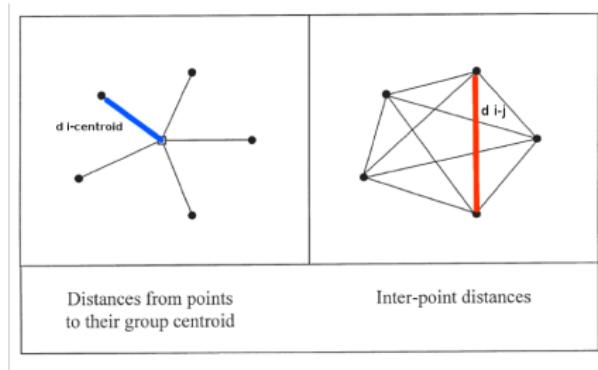
F-distribution





- ▶ Partitioning into variance components:  
 $SS_{total} = SS_{group} + SS_{residual}$
- ▶ **centroids**
- ▶ **p-value by permutations**

- ▶ We can use any distance matrix to partition the variance
- ▶ Sum of squared distances from individual points to their centroid equals the sum of squared interpoint distances divided by the number of points.



$$\sum d_{i\text{-}centroid}^2 = \frac{1}{n} \sum d_{i\text{-}j}^2$$

# PERMANOVA

- ▶ Distance to similarity  $D = [D_{hi}]$ ,  $A = -\frac{1}{2}[D_{hi}^2]$
- ▶ Gower centering (1966).

$$G = \left(I - \frac{11'}{n}\right)A\left(I - \frac{11'}{n}\right)$$

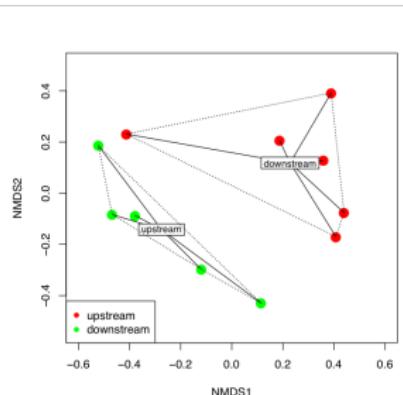
- ▶  $F = \frac{\text{tr}(HGH)}{\text{tr}(I-H)G(I-H)}$ , with  $H = X(X'X)^{-1}X'$
- ▶ Testing via permutations

## Assumptions behind PERMANOVA

- ▶ Equal dispersions
- ▶ Visual inspection

## Pros and Cons

- ▶ Very powerful and easy to implement  
(adnois in *vegan* package)
- ▶ Extension to other studies more difficult
- ▶ Multiple distance test is challenging



# Kernel Machine Regression—A Similarity based Approach

$$y = \mathbf{X}\beta + h(\mathbf{Z}) + \varepsilon$$

where  $h(\cdot)$  is a function in a function space  $\mathcal{H}_K$  generated by positive semidefinite kernel function  $K(\cdot, \cdot)$

- ▶  $\phi_1, \dots, \phi_J$  ( $J$  might be infinite) form a basis of  $\mathcal{H}_K$ , then

$$h(\mathbf{Z}_i) = \sum_{j=1}^J \phi_j(\mathbf{Z}_i) \gamma_j = \phi(\mathbf{Z}_i) \boldsymbol{\gamma},$$

- ▶ Dual representation:  $h(\mathbf{Z}_i) = \sum_{i'=1}^n \alpha_{i'} K(\mathbf{Z}_i, \mathbf{Z}_{i'}).$
- ▶ Simple linear kernel  $K(\mathbf{Z}_i, \mathbf{Z}_{i'}) = \sum_{j=1}^p Z_{ij} Z_{i'j} \Rightarrow \phi_j(\mathbf{Z}) = \mathbf{Z}_j$

## Kernels Specific to Microbiome Data

Key Idea: use the microbiome distance metrics to define similarities.

$$\mathbf{K} = -\frac{1}{2}\left(\mathbf{I} - \frac{\mathbf{1}\mathbf{1}'}{n}\right)\mathbf{D}^2\left(\mathbf{I} - \frac{\mathbf{1}\mathbf{1}'}{n}\right)$$

## Kernels Specific to Microbiome Data

Key Idea: use the microbiome distance metrics to define similarities.

$$\mathbf{K} = -\frac{1}{2}\left(\mathbf{I} - \frac{\mathbf{1}\mathbf{1}'^T}{n}\right)\mathbf{D}^2\left(\mathbf{I} - \frac{\mathbf{1}\mathbf{1}'}{n}\right)$$

## Kernels for Microbiome Data

- ▶ UniFrac distance → UniFrac kernel
  - ▶ Fraction of branches of the phylogenetic tree present in sample  $i$  or sample  $i'$ , but not both
- ▶ Weighted UniFrac distance → Weighted UniFrac kernel
  - ▶ Incorporate abundance information
- ▶ Others: composite distances, Bray-Curtis, Rao's root of patristic distances, etc...

## Testing $h(\mathbf{Z}) = 0$

- ▶ KMR is equivalent to linear mixed model

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{h} + \boldsymbol{\varepsilon}$$

$\mathbf{h} \sim F$  with mean 0 and variance  $\tau \mathbf{K}$

- ▶  $H_0 : h(\mathbf{Z}) = 0 \Leftrightarrow H_0 : \tau = 0.$
- ▶ Variance component statistic:  $Q = (\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}})' \mathbf{K} (\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}}) / \hat{\sigma}^2$
- ▶ Under  $H_0$ ,  $Q$  asymptotically follows a mixture of  $\chi^2$  distribution
- ▶ Small Sample Exact Distribution (*Chen et al., Gen Epi 2016*)

## Equivalent to PERMANOVA

- ▶ No additional covariates
- ▶  $\mathbf{K}$  corresponds to  $\mathbf{D}$

## Omnibus Test

- ▶ The previous model uses one kernel
- ▶ Different  $K$ s are targeted toward different scenarios

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- ▶ The previous model uses one kernel
- ▶ Different  $K$ s are targeted toward different scenarios

## Multiple Kernel Testing

- ▶ Construct single-kernel testing and get their p-values  $p_k$
- ▶ Use  $p^o = \min_{1 \leq k \leq l} p_k$  as the test statistic
- ▶ A residual permutation approach to obtain the null distribution of  $p^o$  considering the correlations
- ▶ Compare  $p^o$  with  $p_1^o, \dots, p_b^o, \dots, p_B^o$  for the final p-value

## Simulations

- ▶ Simulated 850 OTUs using multivariate dirichlet model with parameters estimated from real data
- ▶

$$\mathbf{y} = \boldsymbol{\alpha}' \mathbf{X} + \boldsymbol{\beta}' h(\mathbf{Z}) + \boldsymbol{\epsilon}$$

$X$  is simulated as a confounder

- ▶ Tests:
  - ▶ Individual kernel testing
  - ▶ Omnibus testing
  - ▶ Bonferroni corrected minP method

## Simulation: Power

