

# Metagenome Analysis of Premature Birth

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# Overview

- 1 Introduction
- 2 Materials
- 3 Methods
- 4 Results
- 5 Discussion

# Introduction

# Microbiome

- Microbiota: the microorganisms which live inside & on humans (Turnbaugh et al., 2007)
- Microbiome:  $10^{13}$  to  $10^{14}$  microorganisms whose collective genome (Gill et al., 2006)



**Figure:** Concept of a core human microbiome (Turnbaugh et al., 2007)

- Ribosomal RNA
- Well-known as a key to phylogeny (Olsen & Woese, 1993)

# Premature Birth (Preterm Birth; PTB)

PTB:

- ①  $\text{PTB} < 37 \text{ GW}$  (Gestational week)
- ②  $\text{Normal} \geq 37 \text{ GW}$

Detailed PTB:

- ① Extremely PTB  $< 28 \text{ GW}$
- ②  $28 \text{ GW} \leq \text{Very PTB} < 34 \text{ GW}$
- ③  $34 \text{ GW} \leq \text{Late PTB} < 37 \text{ GW}$
- ④  $\text{Normal} \geq 37 \text{ GW}$

(J. Tucker & McGuire, 2004; Voronkov, Solonovych, Liashenko, & Revenko, 2018)

# Materials

# 16S rRNA Sequencing

**16S rRNA sequencing** is the *reference method* for bacterial taxonomy & identification (Mignard & Flandrois, 2006)

Three main reasons (Janda & Abbott, 2007):

- 16S rRNA exists in almost all bacteria
- Functions of the 16S rRNA has not changed over time
- 16S rRNA is large enough for bioinformatics



# Train/Test Data vs. Validate Data

- JBNU/Helixco data
  - First data
  - Second data
  - Stool data

Table: Sample Information

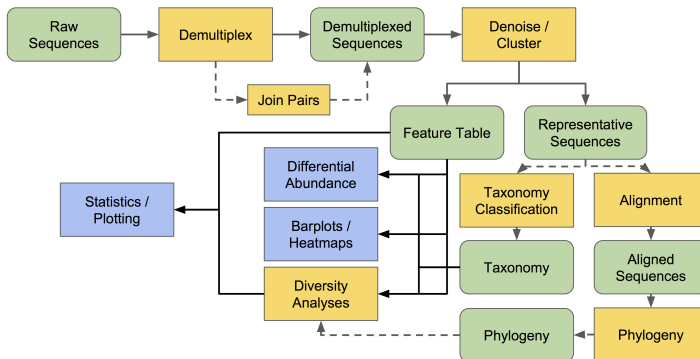
Data	Participants	Samples	Remarks
First	24	107	-
Second	35	288	-
Third	10	106	-
Stool	63	126	Stool

# Methods

# Methods

## Qiime 2 Workflow

# Qiime 2 Workflow



**Figure:** QIIME 2 workflow (Bolyen et al., 2019; Mandal, Van Treuren, White, Eggesbø, et al., 2015; McDonald et al., 2012)

# Filtering with Quality Score

Drawback between:

- Longer sequence read
- Higher quality value

∴ Select the maximum length  $n$  where:

$$\begin{aligned} \forall n_i \in \{n_k | \text{MedianQualityScore} \geq 30\} \\ \exists ! n \in \{n_i\} : n \geq n_i \end{aligned} \tag{1}$$

# Denoising Techniques

- DADA2: Amplicon Sequence Variants (ASVs) (Callahan et al., 2016)
- Deblur: Operational Taxonomic Units (OTUs) (Amir et al., 2017)

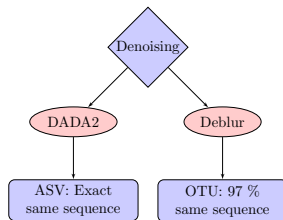


Figure: Denoising Algorithms

# Taxonomy Classification

- Greengenes (GG) (DeSantis et al., 2006)
- SILVA (Pruesse et al., 2007; Quast et al., 2012)

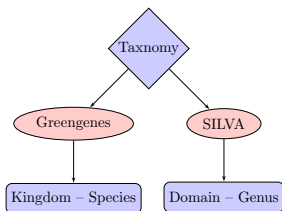


Figure: Taxonomy Classifications

“A **higher** performance at taxonomic levels above *genus level*;  
but performance appears to **drop** at *species level*” (Gihawi et al., 2019)

# Merging Denoising/Taxonomy

Merging multiple IDs (ASVs or OTUs) into one, which have

- Different IDs
- Identified as same taxonomy



Figure: Example Diagram for Merging Denoising/Taxonomy



# Methods

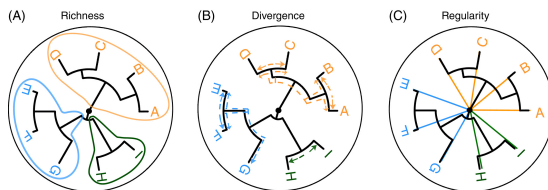
## Abundance Test

- Analysis of composition of microbiome (Mandal, Van Treuren, White, Eggesbø, et al., 2015)
- ANCOM detects significantly abundant taxa, while maintain high statistical power
- Find taxa that can divide each classes

# Methods

## Diversity Indices

# Diversity Indices



**Figure:** Three dimensions of phylogenetic information (C. M. Tucker et al., 2017)

- A quantitative measure that shows richness, divergence, and regularity (C. M. Tucker et al., 2017)
- Alpha diversity indices: the richness of taxa **at a single community**
- Beta diversity indices: the taxonomic differentiation **between communities**

# Alpha Diversity Indices

- Evenness index
- Faith's Phylogenetic Diversity (Faith PD) index
- Observed Features index
- Shannon's Diversity index

# Beta Diversity Indices

- Bray-Curtis distance index
- Jaccard distance index
- Unweighted UniFrac distance index
- Weighted UniFrac distance index

Methods

Miscellaneous

# t-distributed Stochastic Neighbor Embedding (t-SNE)



Figure: t-SNE with handwritten data (Maaten & Hinton, 2008)



- Pandas (McKinney et al., 2011)
- Scikit-Learn (Pedregosa et al., 2011)
- SciPy (Virtanen et al., 2020)
- Matplotlib (Hunter, 2007)
- Seaborn (Waskom et al., 2020)
- Statannot

## Results

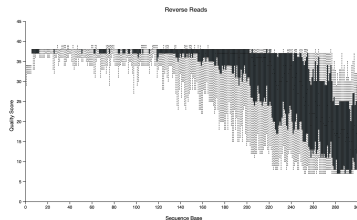
# Results

## Filtering Results

# Quality Score from JBNU/Helixco Data



(a) Forward



(b) Reverse

Figure: Quality Score Plot

- $\ell_{Forward} = 300$
- $\ell_{Reverse} = 245$

# Results

## t-SNE with Clinical Information

# Workflow for t-SNE with Site/Premature Information

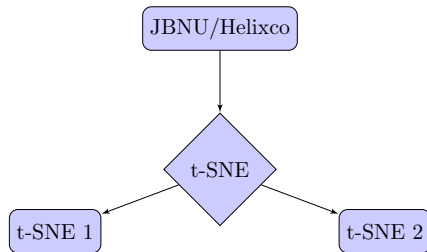


Figure: Workflow of t-SNE for Site/Premature Information

- Diseases
  - ① Gestational Diabetes
  - ② Maternal Overweight/Obesity
  - ③ Maternal Weight Gain
  - ④ Hypertension
  - ⑤ PROM
  - ⑥ Antibiotic
  - ⑦ Steroid
- Probing sites
  - ① Maternal Mouth

# Selected t-SNE Plots I

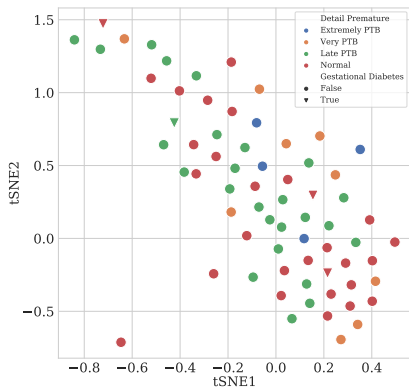


Figure: t-SNE about Gestational Diabetes



# Selected t-SNE Plots II



(a) Overweight



(b) Weight gain



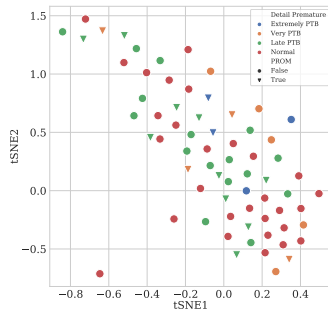
(c) Too much gain

Figure: t-SNE about Maternal Weight

# Selected t-SNE Plots III



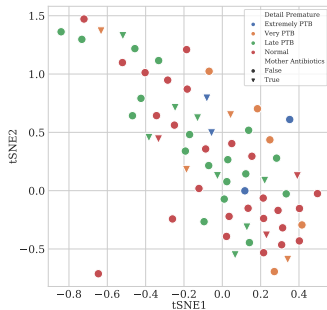
(a) Hypertension



(b) PROM

Figure: t-SNE about Disease

# Selected t-SNE Plots IV



(a) Mother



(b) Neonate

Figure: t-SNE about Antibiotics Usage

# Selected t-SNE Plots V

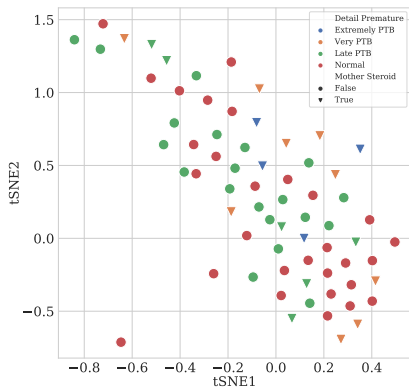


Figure: t-SNE about Steroid Usage

# Results

## Bacterial Abundance Test with ANCOM

- Analysis of composition of microbiomes
- ANCOM can be used for analyzing the composition of microbiomes in multiple populations (Mandal, Van Treuren, White, Eggesbø, et al., 2015)
- Differential abundance testing
- ① clr: Centered log(*Ratio*)
- ② W: a count of the number of sub-hypothesis which have passed for given species

Site selection:

- 1 Neonatal mouth: 1-day, 3-day, and 5-day
- 2 Cervix
- 3 Maternal mouth
- 4 Vagina

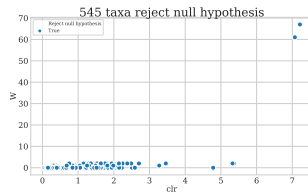
PTB:

- 1 Premature
- 2 Detail premature

# ANCOM with Neonatal Mouth



(a) PTB

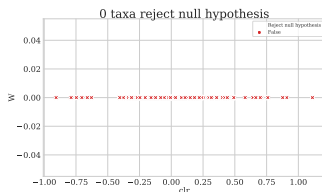


(b) Detailed PTB

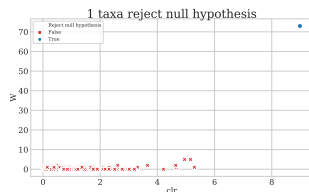
Figure: ANCOM with Neonatal Mouth



# ANCOM with Maternal Mouth



(a) PTB

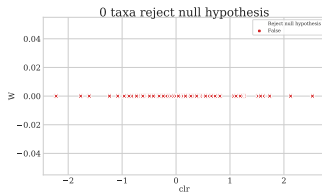


(b) Detailed PTB

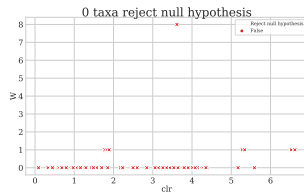
Figure: ANCOM with Maternal Mouth

- *Bacteria Proteobacteria Alphaproteobacteria Rickettsiales mitochondria* family

# ANCOM with Cervix



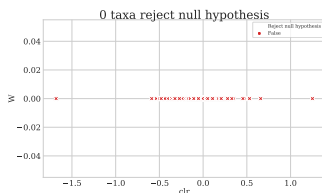
(a) PTB



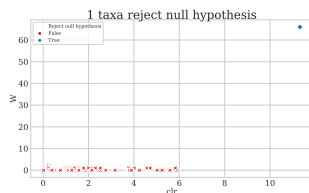
(b) Detailed PTB

Figure: ANCOM with Cervix

# ANCOM with Vagina



(a) PTB



(b) Detailed PTB

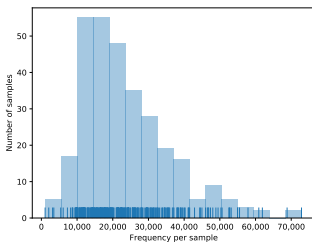
Figure: ANCOM with Vagina

- *Bacteria Proteobacteria Epsilonproteobacteria Campylobacterales Campylobacteraceae Campylobacter genus*

## Results

### Diversity Index

# Rarefaction



(a) DADA2



(b) Deblur

Figure: Rarefaction from the Data

- $\min(\ell_{DADA2})$ : 1046
- $\min(\ell_{Deblur})$ : 4864

# Diversity Indices

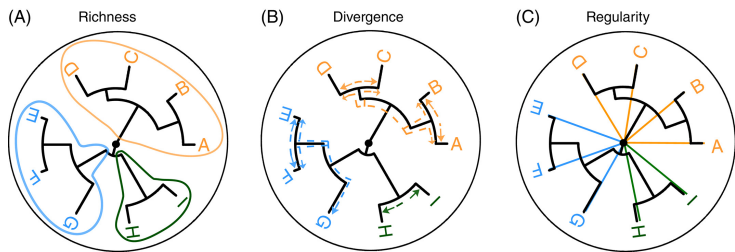
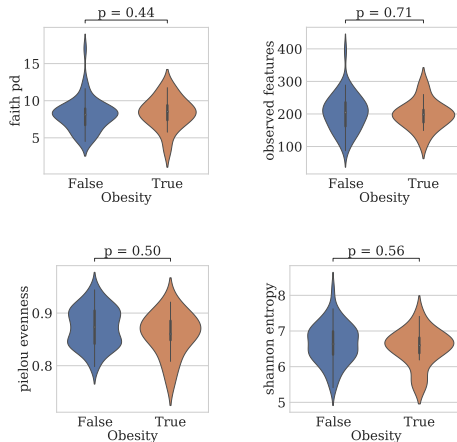


Figure: Dimensions of phylogenetic information (C. M. Tucker et al., 2017)

- Alpha-diversity: the species diversity *in* a local scale
- Beta-diversity: the species diversity *between* local scales

- Alpha diversity indices
  - ① Faith PD
  - ② Observed Features
  - ③ Pielou Evenness
  - ④ Shannon Entropy
- Diseases/Conditions
  - ① Gestational Diabetes
  - ② Too much Weight Gain
  - ③ Overweight/Obesity
  - ④ Hypertension
  - ⑤ PROM
  - ⑥ Antibiotics
  - ⑦ Steroid
- Site Selection
  - ① Maternal Mouth
  - ② Neonatal Mouth - 3 day

# Alpha-diversity Violin Plots I



**Figure:** Alpha-diversities by DADA2 & Overweight/Obesity in Maternal Mouth



# Alpha-diversity Violin Plots II

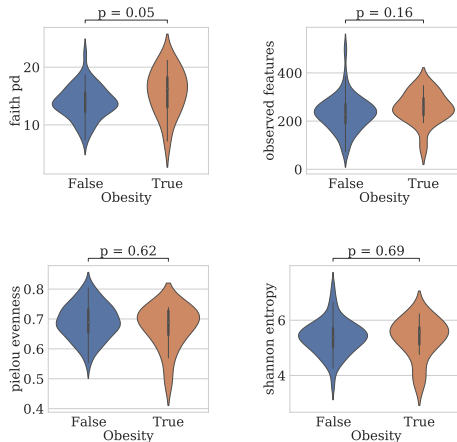


Figure: Alpha-diversities by Deblur & Overweight/Obesity in Maternal Mouth

# Alpha-diversity Violin Plots III

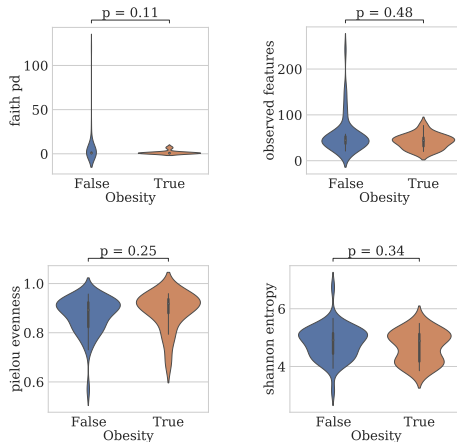


Figure: Alpha-diversities by DADA2 & Overweight/Obesity in Neonatal-3day

# Alpha-diversity Violin Plots IV

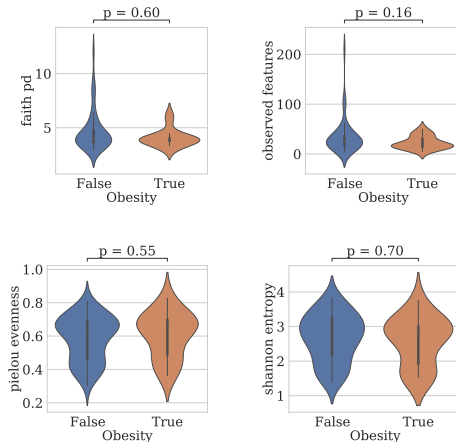


Figure: Alpha-diversities by Deblur & Overweight/Obesity in Neonatal-3day

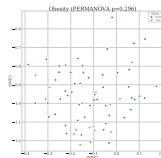
# Beta-diversity

- Beta diversity indices
  - ① Bray-Curtis distance
  - ② Jaccard distance
  - ③ Unweighted UniFrac distance
  - ④ Weighted UniFrac distance
- Diseases/Conditions
  - ① Gestational Diabetes
  - ② Too much Weight Gain
  - ③ Overweight/Obesity
  - ④ Hypertension
  - ⑤ PROM
  - ⑥ Antibiotics
  - ⑦ Steroid
- Site Selection
  - ① Maternal Mouth
  - ② Neonatal Mouth - 3 day

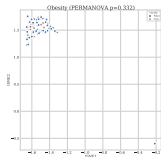
# Beta-diversity Scatter Plots I



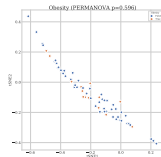
(a) Bray-Curitis



(b) Jaccard



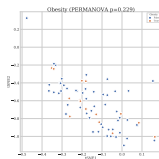
(c) Unweighted UniFrac



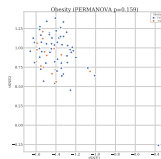
(d) Weighted UniFrac

Figure: Beta-diversities by DADA2 & Overweight/Obesity in Maternal Mouth

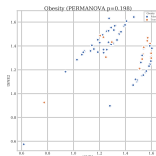
# Beta-diversity Scatter Plots II



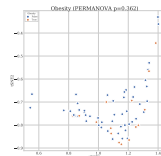
(a) Bray-Curitis



(b) Jaccard



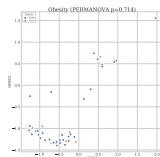
(c) Unweighted UniFrac



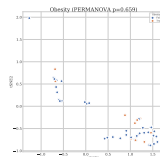
(d) Weighted UniFrac

**Figure:** Beta-diversities by Deblur & Overweight/Obesity in Maternal Mouth

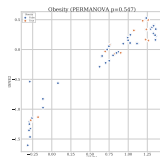
# Beta-diversity Scatter Plots III



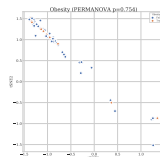
(a) Bray-Curitis



(b) Jaccard



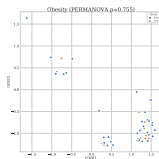
(c) Unweighted UniFrac



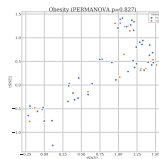
(d) Weighted UniFrac

Figure: Beta-diversities by DADA2 & Overweight/Obesity in Neonate 3-day

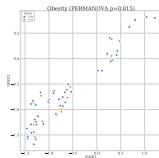
# Beta-diversity Scatter Plots IV



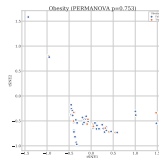
(a) Bray-Curtis



(b) Jaccard



(c) Unweighted UniFrac



(d) Weighted UniFrac

**Figure:** Beta-diversities by Deblur & Overweight/Obesity in Neonate 3-day



# Results

## Metagenomic Biomarker Discovery

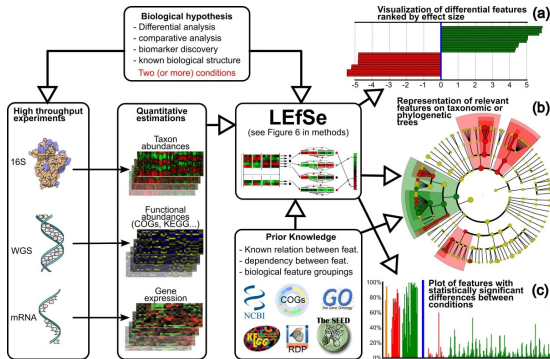


Figure: LEfSe workflow (Segata et al., 2011)

- Analysis Pipeline
  - Algorithm: DADA2 and Deblur
  - Databases: GG, Silva, and HOMD
- Subject Groups
  - PTB
  - Normal
- Sites
  - Cervix
  - Mouth
  - Neonatal Mouth
  - Vagina

# LEfSe Results with PTB I

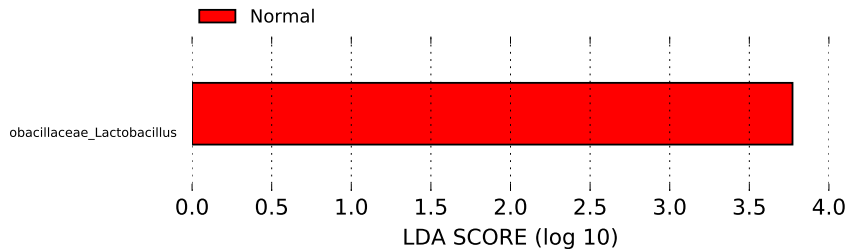
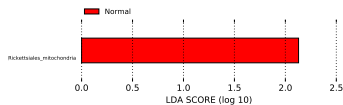
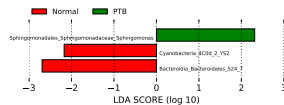


Figure: Deblur+HOMD in Cervix

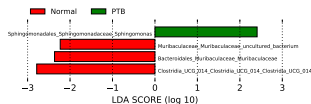
# LEfSe Results with PTB II



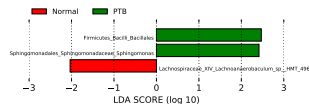
(a) DADA2+GG



(b) Deblur+GG



(c) Deblur+Silva



(d) Deblur+HOMD

Figure: LEfSe in Neonate 1-day

# LEfSe Results with PTB III

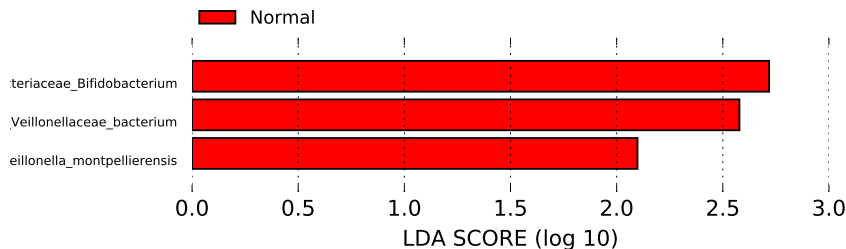
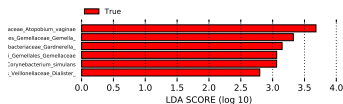
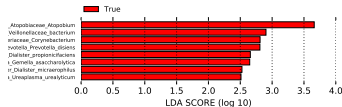


Figure: Deblur+Silva in Vagina

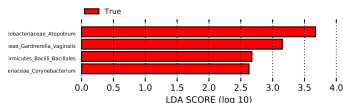
# LEfSe Results with Obesity I



(a) Deblur+GG



(b) Deblur+Silva



(c) Deblur+HOMD

Figure: LEfSe in Cervix

# LEfSe Results with Obesity II

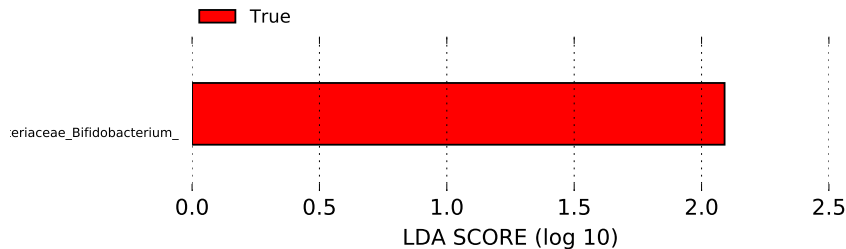


Figure: DADA2+GG in Vagina



## Discussion

- Human Oral Microbiome Database
- LEfSe: explain differences between classes for statistical significance

- Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Xu, Z. Z., ... others (2017). Deblur rapidly resolves single-nucleotide community sequence patterns. *MSystems*, 2(2).
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using qiime 2. *Nature Biotechnology*, 37(8), 852-857. Retrieved from <https://doi.org/10.1038/s41587-019-0209-9> doi: 10.1038/s41587-019-0209-9
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). Dada2: high-resolution sample inference from illumina amplicon data. *Nature methods*, 13(7), 581-583.

- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ... Andersen, G. L. (2006). Greengenes, a chimera-checked 16s rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*, 72(7), 5069–5072.
- Gihawi, A., Rallapalli, G., Hurst, R., Cooper, C. S., Leggett, R. M., & Brewer, D. S. (2019). Sepath: benchmarking the search for pathogens in human tissue whole genome sequence data leads to template pipelines. *Genome biology*, 20(1), 1–15.
- Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., ... Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *science*, 312(5778), 1355–1359.
- Hunter, J. D. (2007). Matplotlib: A 2d graphics environment. *Computing in science & engineering*, 9(3), 90–95.

# References III

- Janda, J. M., & Abbott, S. L. (2007). 16s rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, 45(9), 2761–2764.
- Maaten, L. v. d., & Hinton, G. (2008). Visualizing data using t-sne. *Journal of machine learning research*, 9(Nov), 2579–2605.
- Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease*, 26(1), 27663.
- Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease*, 26(1), 27663. doi: 10.3402/mehd.v26.27663

- McDonald, D., Clemente, J. C., Kuczynski, J., Rideout, J. R., Stombaugh, J., Wendel, D., ... Caporaso, J. G. (2012). The biological observation matrix (biom) format or: how i learned to stop worrying and love the ome-ome. *GigaScience*, 1(1), 7. doi: 10.1186/2047-217X-1-7
- McKinney, W., et al. (2011). pandas: a foundational python library for data analysis and statistics. *Python for High Performance and Scientific Computing*, 14(9).
- Mignard, S., & Flandrois, J.-P. (2006). 16s rna sequencing in routine bacterial identification: a 30-month experiment. *Journal of microbiological methods*, 67(3), 574–581.
- Olsen, G. J., & Woese, C. R. (1993). Ribosomal rna: a key to phylogeny. *The FASEB journal*, 7(1), 113–123.

- Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., ... others (2011). Scikit-learn: Machine learning in python. *the Journal of machine Learning research*, 12, 2825–2830.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., & Glöckner, F. O. (2007). Silva: a comprehensive online resource for quality checked and aligned ribosomal rna sequence data compatible with arb. *Nucleic acids research*, 35(21), 7188–7196.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... Glöckner, F. O. (2012). The silva ribosomal rna gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), D590–D596.
- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome biology*, 12(6), 1–18.

- Tucker, C. M., Cadotte, M. W., Carvalho, S. B., Davies, T. J., Ferrier, S., Fritz, S. A., ... others (2017). A guide to phylogenetic metrics for conservation, community ecology and macroecology. *Biological Reviews*, 92(2), 698–715.
- Tucker, J., & McGuire, W. (2004). Epidemiology of preterm birth. *Bmj*, 329(7467), 675–678.
- Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature*, 449(7164), 804–810.
- Virtanen, P., Gommers, R., Oliphant, T. E., Haberland, M., Reddy, T., Cournapeau, D., ... others (2020). Scipy 1.0: fundamental algorithms for scientific computing in python. *Nature methods*, 17(3), 261–272.



- Voronkov, L., Solonovych, A., Liashenko, A., & Revenko, I. (2018). Prognostic value of cognitive tests and their combination in patients with chronic heart failure and reduced left ventricular ejection fraction. *Eureka: health sciences*(6), 36–45.
- Waskom, M., Botvinnik, O., Ostblom, J., Gelbart, M., Lukauskas, S., Hobson, P., ... Brian (2020, April). *mwaskom/seaborn: v0.10.1 (april 2020)*. Zenodo. Retrieved from <https://doi.org/10.5281/zenodo.3767070> doi: 10.5281/zenodo.3767070