Methods in Microbiota Research: Part II

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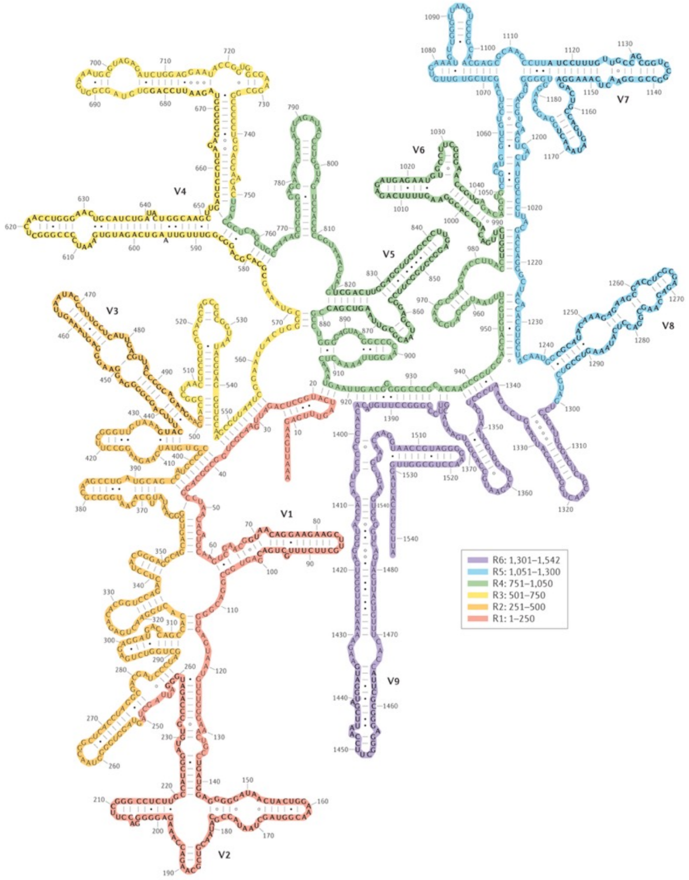
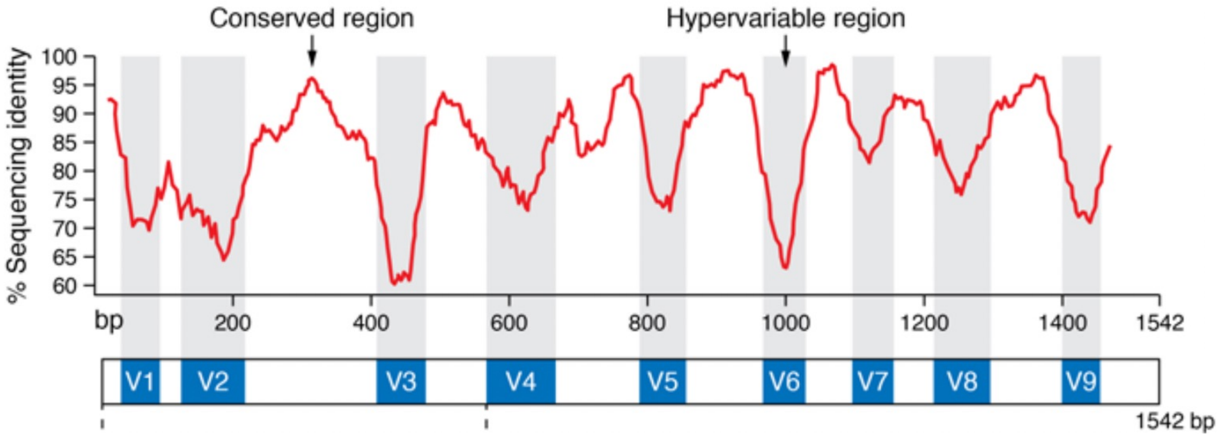
Notes prior to beginning

- Google is a coder’s best friend
  - Almost every error message has a solution on some online forum
- Statistical modeling/testing in microbiome science is a constantly evolving area
  - Precedent does not always justify usage – methods may be outdated
  - No consensus on best methods – more clarity on what not to do
- I am self-taught
  - If I can do it, so can you!

Tools of the Trade

The bacterial 16S ribosomal RNA gene as a phylogenetic barcode





Tools of the Trade

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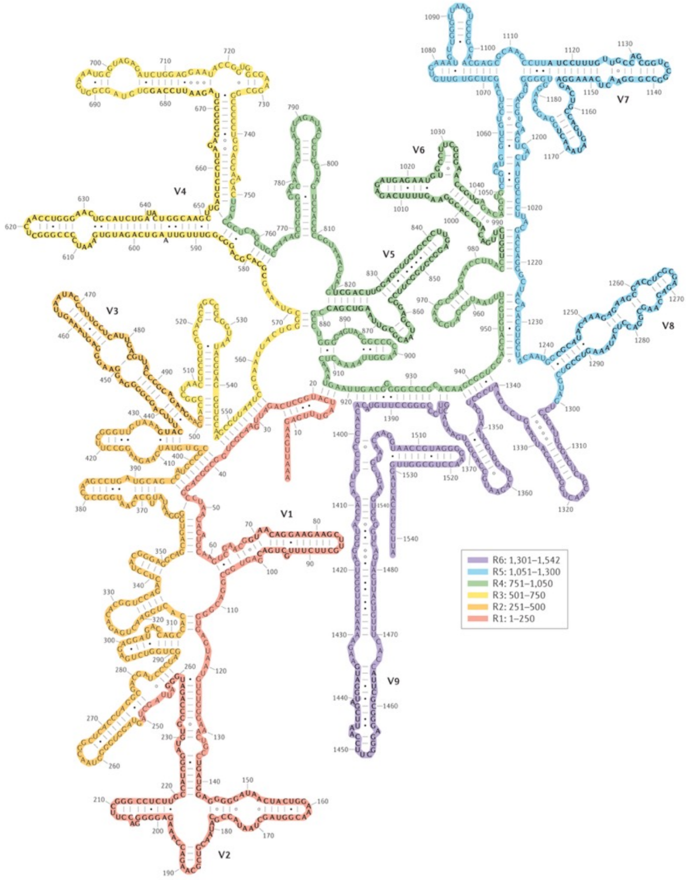
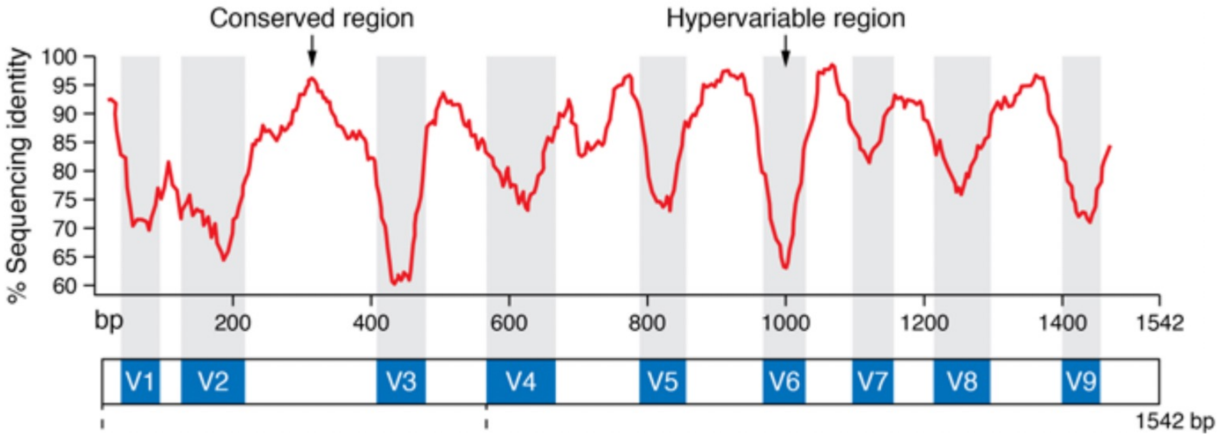
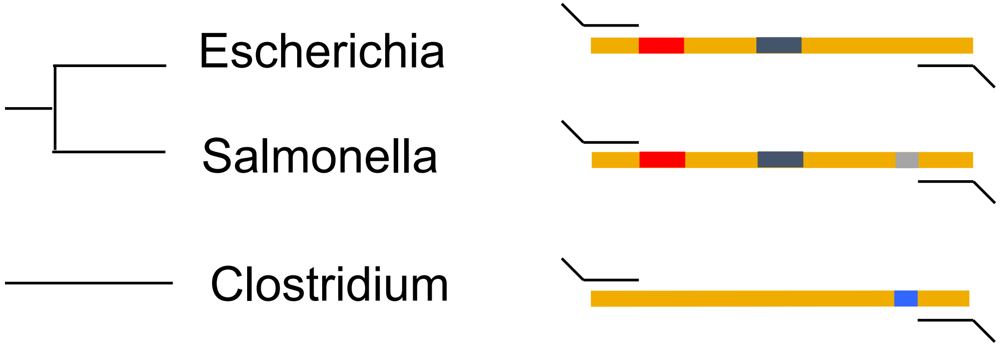






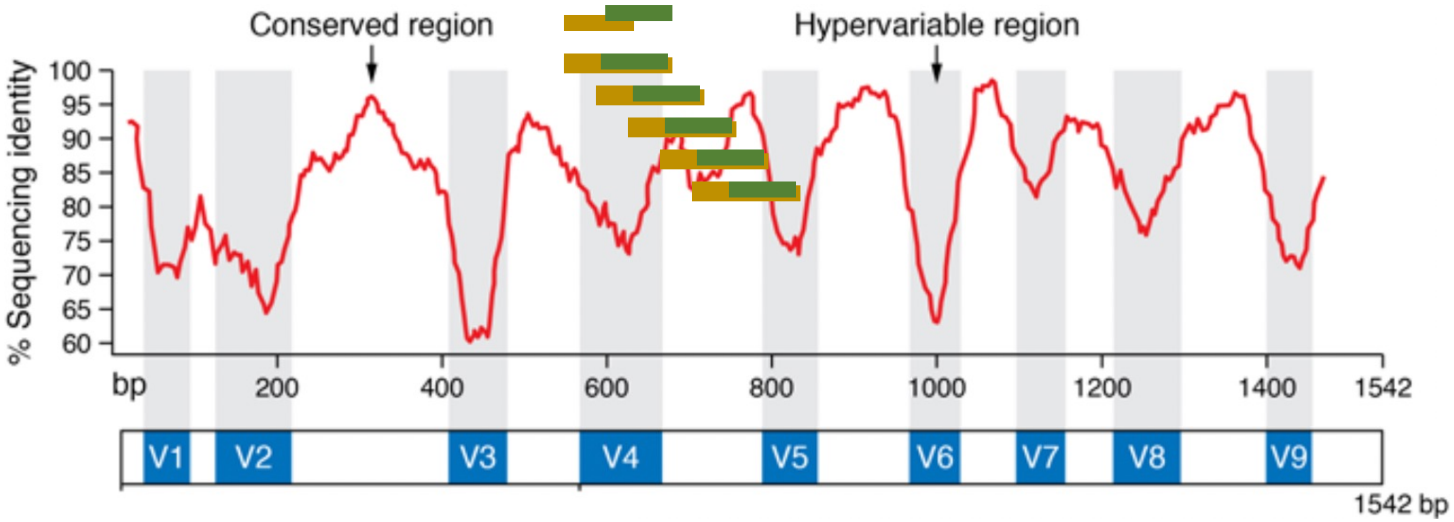
Escherichia

Salmonella

Clostridium

- Amplification and high-throughput sequencing allows quantification of microbiota constituents

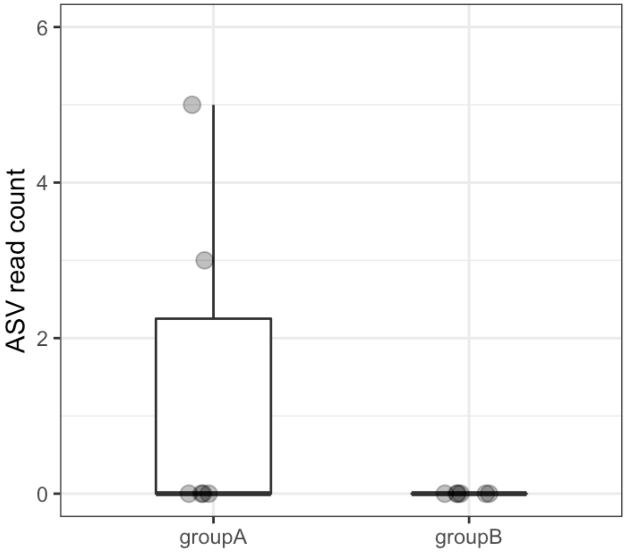
Pre-processing: Chimera filtering

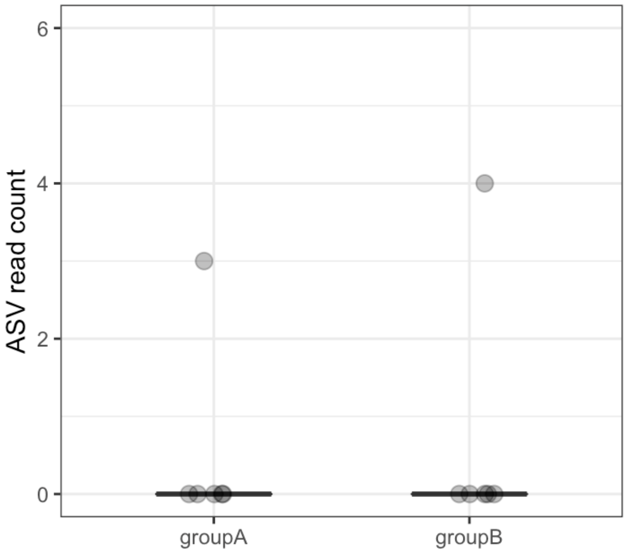


- Chimeras are identifiable if one portion is identical to a more abundant ‘parent’ sequence and the rest is identical to another ‘parent’ sequence
  - ‘removeBimeraDenovo’ function in dada2

Pre-processing:
removing taxa with low read counts

Mean fold
difference
A/B
= infinity

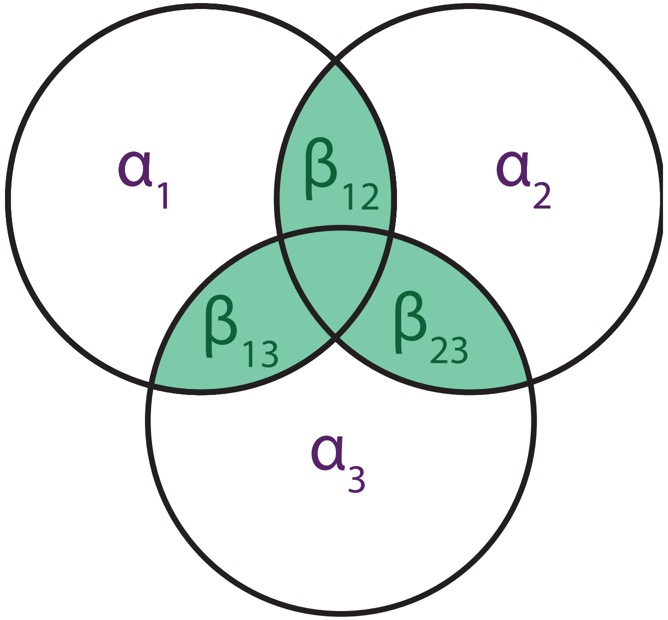




Mean fold
difference
A/B
= 0.75

- Random re-sampling (e.g. multiple rarefactions or re-sequencing) of samples causes wide variation in abundance of taxa with low read counts
- Fold changes between groups can be very unstable and unreliable
  - Example solution: filter taxa that are below 0.001% abundance threshold

Comparative metrics: alpha vs. beta diversity



Alpha diversity:

- The number of different taxa present in a sample (richness) and/or the relative distribution of these taxa (evenness)
  - Examples: chao1 (richness), Shannon or inverse Simpson (richness and evenness), Pielou’s (evenness)
- Each sample gets its own alpha diversity quantification

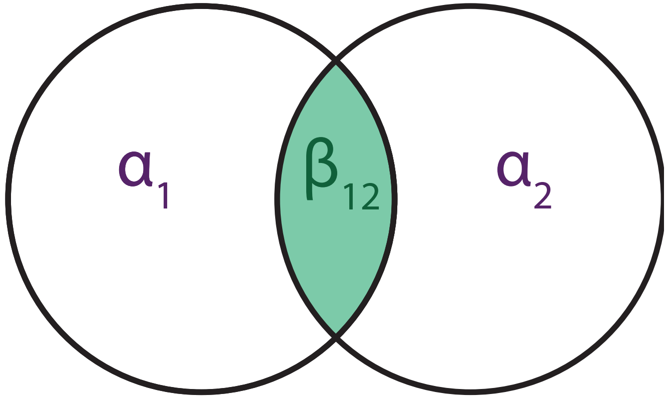
Beta diversity:

- The ecological similarity *between two samples*
  - Similarity in common taxon membership (ex. Canberra, Bray-Curtis), phylogenetic similarity of taxa within a community (ex. Unweighted UniFrac)
- Each *pair of samples* gets its own beta diversity quantification

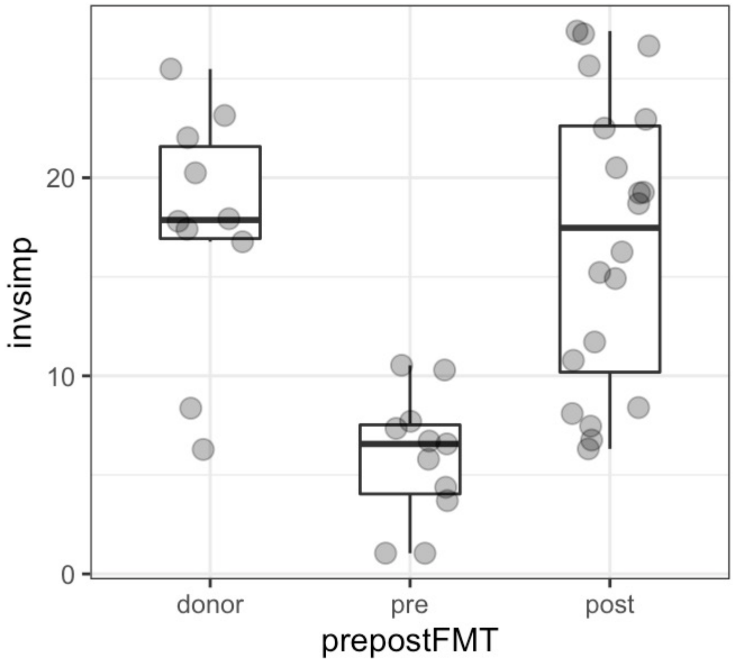
Beta diversity matrix

Subject ID	1	2	3	…
1	0	0.22	0.9	
2	0.22	0	0.47	
3	0.9	0.47	0	
…				

Comparative metrics: alpha vs. beta diversity



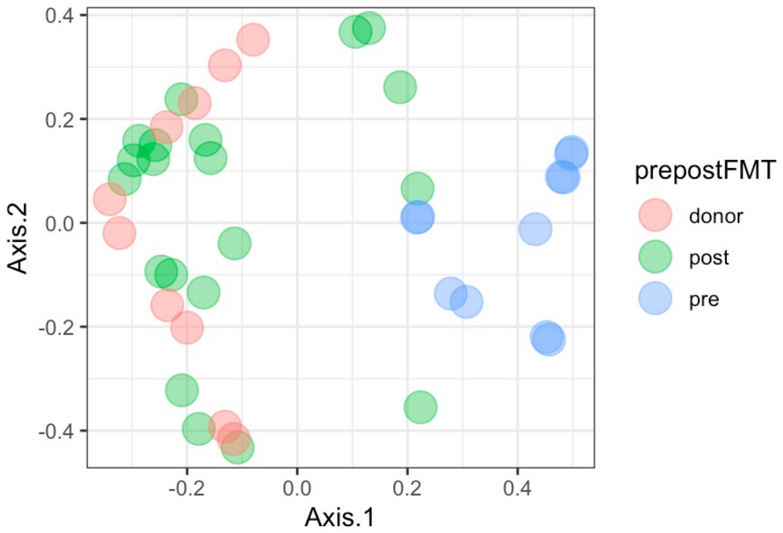
Inverse Simpson
(Alpha diversity)



Beta diversity matrix

Subject ID	1	2	3	…
1	0	0.22	0.9	
2	0.22	0	0.47	
3	0.9	0.47	0	
…				

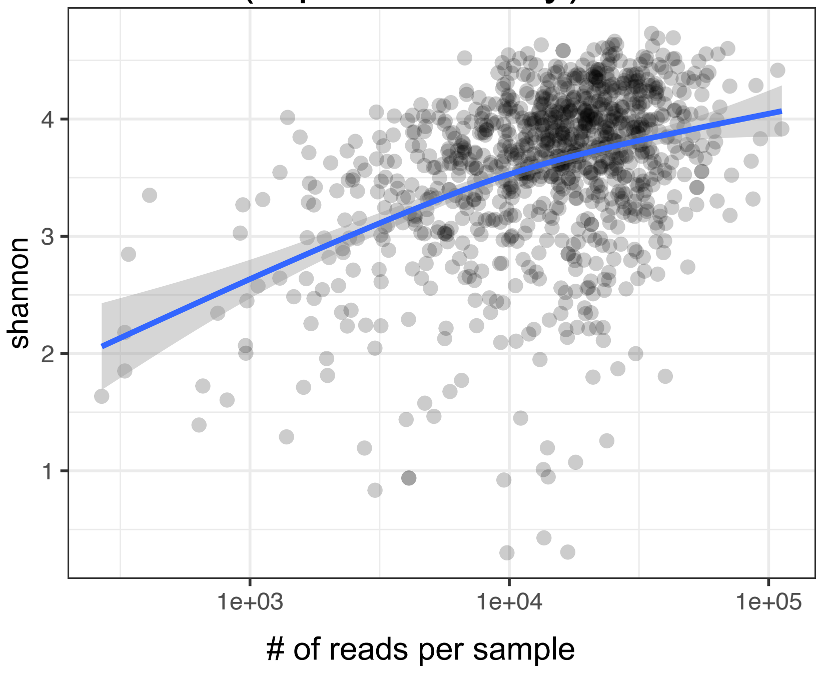
Principal Coordinates Analysis (PCoA)
(Based on beta diversity matrix)



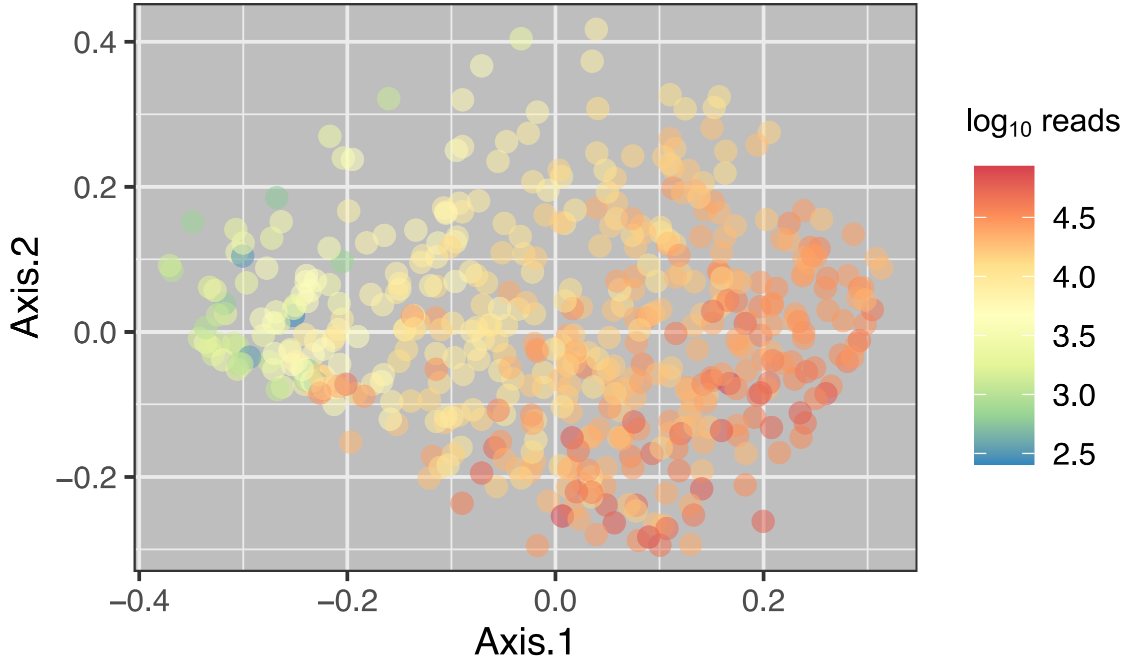
Pre vs. post
PERMANOVA
P = 0.00001

Alpha and beta diversity are skewed by sequencing read depth

Shannon diversity
(Alpha diversity)



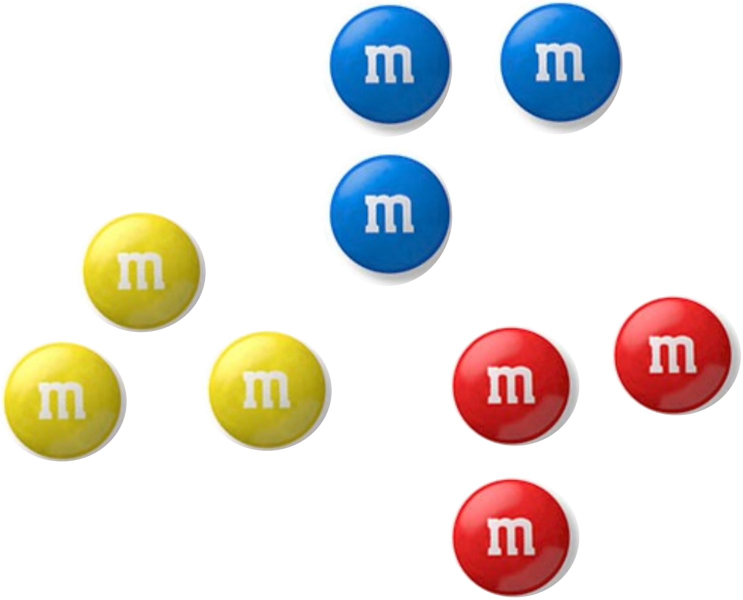
Principal Coordinates Analysis (PCoA)
(Based on beta diversity matrix)



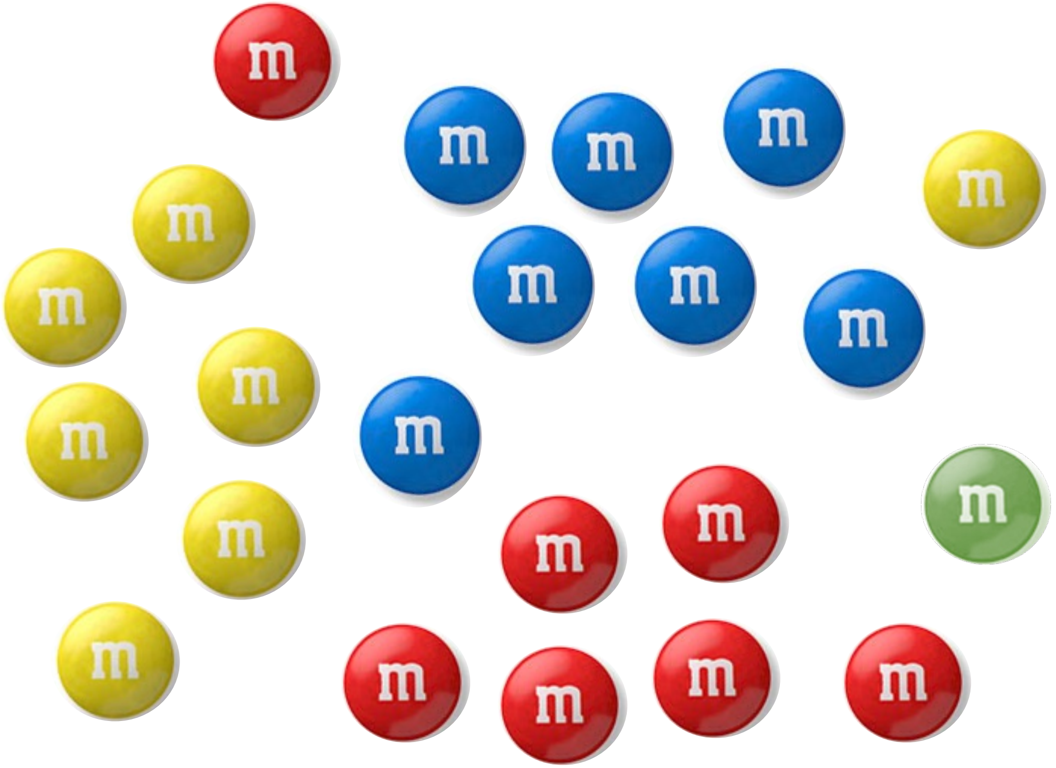
Rarefaction: random selection of ‘n’ reads per sample

Randomly sub-sample 9 candies from each handful
(rarefy to 9 candies)

Handful #1



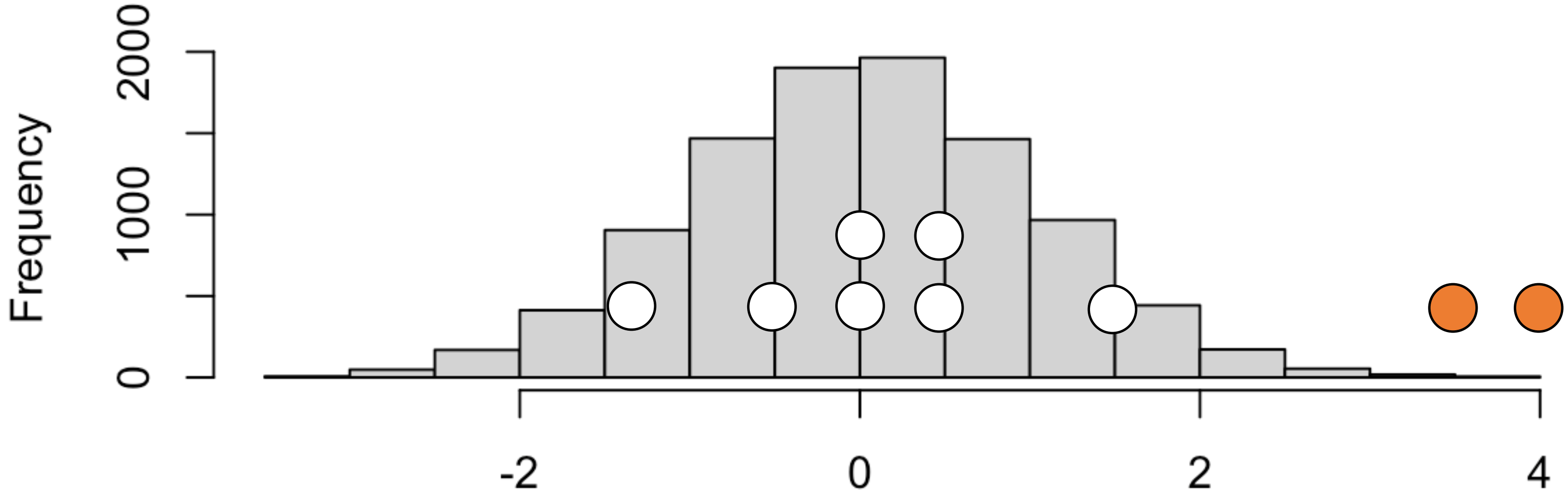
Handful #2



Let’s get coding

Statistical analyses:
Data assumptions

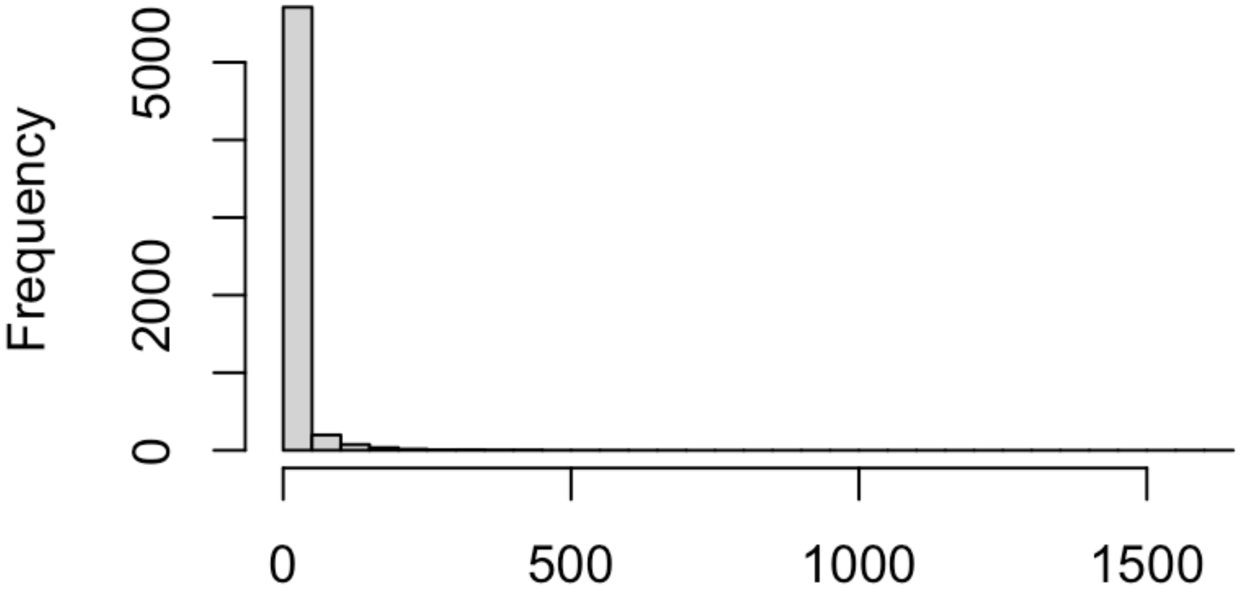
- Parametric statistical tests assume normally distributed data:



- This allows the test to ‘infer’ missing data for groups with low N

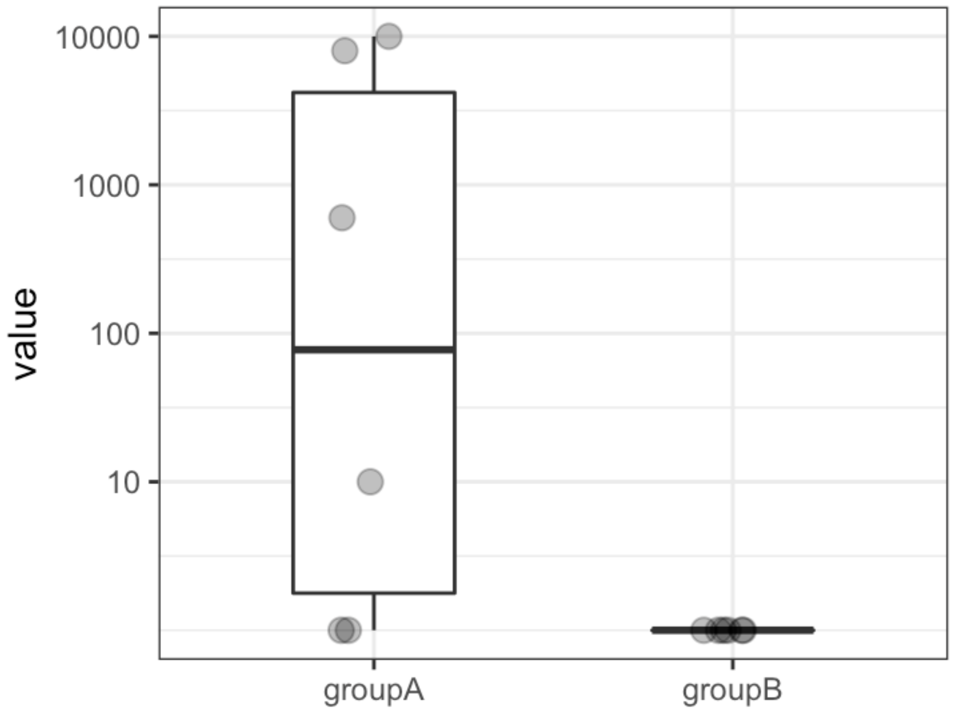
Statistical analyses: Data assumptions

- Parametric statistical tests assume normally distributed data
- Microbiome data are not normally distributed:



Statistical analyses: Data assumptions

- Parametric statistical tests assume normally distributed data
- Microbiome data are not normally distributed:



T-test P = 0.161
(parametric)

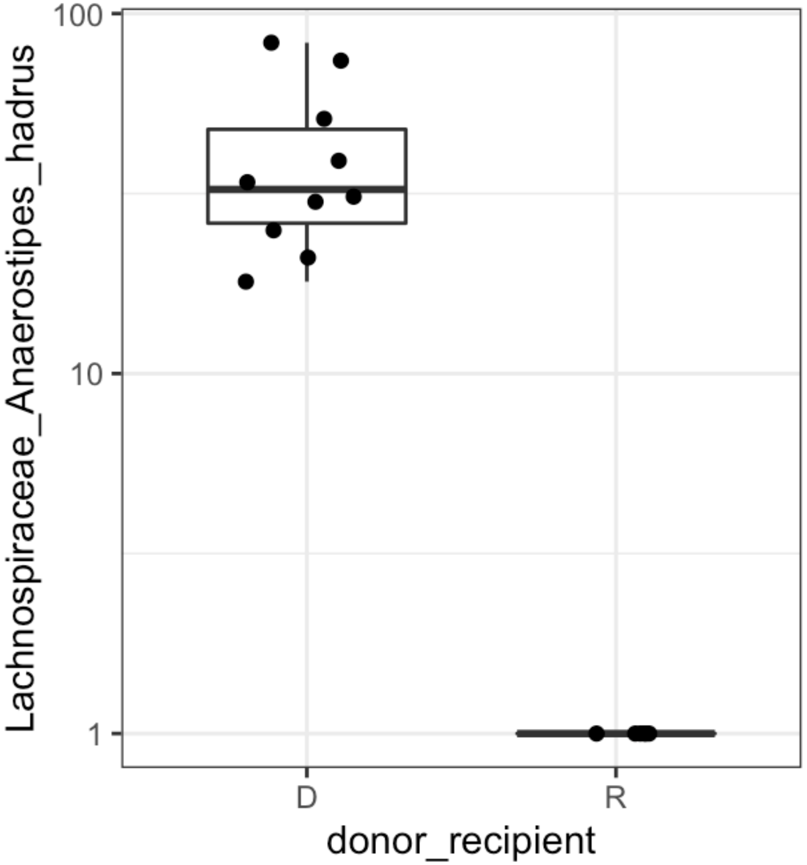
Mann-Whitney P = 0.028
(non-parametric)

Non-parametric tests with dichotomous variables:

- Goal: Identify taxa that are in differential abundance between two groups of samples
- Method: make no assumptions about data distribution using non-parametric statistics
    - non-parametric statistical test for each taxon, successively
        - ex. Mann-Whitney U test/Wilcoxon rank-sum test
    - then perform multiple comparisons corrections
        - ex. Benjamini-Hochberg false discovery rate calculation

Non-parametric tests with dichotomous variables: Results

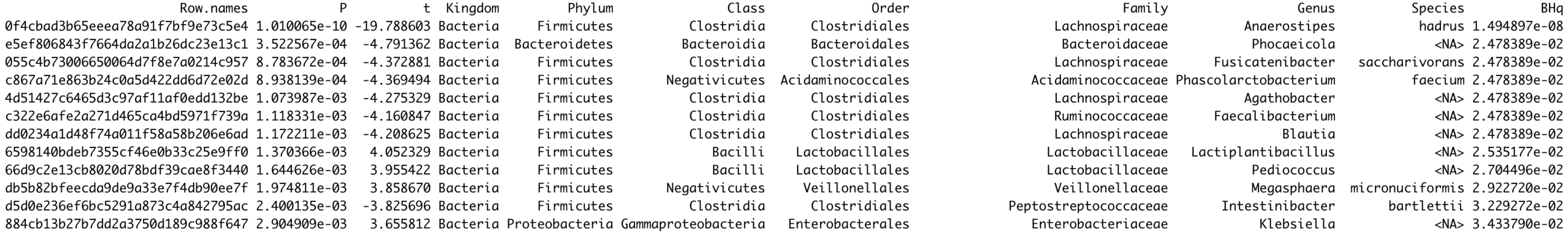
```
                       Row.names      wilx_P wilx_stat     log2fc numnonzero rank      BH_Q  Kingdom         Phylum               Class              Order                            Family                 Genus
0f4cbad3b65eeea78a91f7bf9e73c5e4 0.001018407       0.0       -Inf         26   16 0.0952019 Bacteria     Firmicutes          Clostridia      Clostridiales                   Lachnospiraceae          Anaerostipes
6598140bdeb7355cf46e0b33c25e9ff0 0.005458783      50.0        Inf          7   46 0.0952019 Bacteria     Firmicutes             Bacilli    Lactobacillales                  Lactobacillaceae   Lactiplantibacillus
66d9c2e13cb8020d78bdf39cae8f3440 0.005458783      50.0        Inf          9   25 0.0952019 Bacteria     Firmicutes             Bacilli    Lactobacillales                  Lactobacillaceae           Pediococcus
884cb13b27b7dd2a3750d189c988f647 0.005458783      50.0        Inf          6   19 0.0952019 Bacteria Proteobacteria Gammaproteobacteria   Enterobacterales                Enterobacteriaceae            Klebsiella
```



Controlling for confounding variables in group comparisons: linear mixed effects models

- Goal: Identify taxa that are in differential abundance between two groups of samples and control for confounding variables
- Method: linear mixed effects models
  - Requires linear variables (ideally normally distributed) → convert data to 'linear'-esque space using transformations
    - Ex. limma, arcsin square root, log with pseudocount
  - Perform linear mixed effects with covariates as fixed/random effects
  - Perform multiple comparisons correction
    - ex. Benjamini-Hochberg false discovery rate calculation (FDR 'Q' value)

Controlling for confounding variables in group comparisons: linear mixed effects models



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                       Row.names            P          t  Kingdom         Phylum               Class             Order                            Family                 Genus          Species          BHq
0f4cbad3b65eeea78a91f7bf9e73c5e4 1.010065e-10 -19.788603 Bacteria     Firmicutes          Clostridia     Clostridiales                   Lachnospiraceae          Anaerostipes           hadrus 1.494897e-08
e5ef806843f7664da2a1b26dc23e13c1 3.522567e-04  -4.791362 Bacteria  Bacteroidetes         Bacteroidia     Bacteroidales                    Bacteroidaceae           Phocaeicola             <NA> 2.478389e-02
055c4b73006650064d7f8e7a0214c957 8.783672e-04  -4.372881 Bacteria     Firmicutes          Clostridia     Clostridiales                   Lachnospiraceae      Fusicatenibacter   saccharivorans 2.478389e-02
c867a71e863b24c0a5d422dd6d72e02d 8.938139e-04  -4.369494 Bacteria     Firmicutes       Negativicutes Acidaminococcales                Acidaminococcaceae Phascolarctobacterium          faecium 2.478389e-02
4d51427c6465d3c97af11af0edd132be 1.073987e-03  -4.275329 Bacteria     Firmicutes          Clostridia     Clostridiales                   Lachnospiraceae           Agathobacter             <NA> 2.478389e-02
c322e6afe2a271d465ca4bd5971f739a 1.118331e-03  -4.160847 Bacteria     Firmicutes          Clostridia     Clostridiales                   Ruminococcaceae      Faecalibacterium             <NA> 2.478389e-02
dd0234a1d48f74a011f58a58b206e6ad 1.172211e-03  -4.208625 Bacteria     Firmicutes          Clostridia     Clostridiales                   Lachnospiraceae               Blautia             <NA> 2.478389e-02
6598140bdeb7355cf46e0b33c25e9ff0 1.370366e-03   4.052329 Bacteria     Firmicutes             Bacilli   Lactobacillales                  Lactobacillaceae   Lactiplantibacillus             <NA> 2.535177e-02
66d9c2e13cb8020d78bdf39cae8f3440 1.644626e-03   3.955422 Bacteria     Firmicutes             Bacilli   Lactobacillales                  Lactobacillaceae           Pediococcus             <NA> 2.704496e-02
db5b82bfeecda9de9a33e7f4db90ee7f 1.974811e-03   3.858670 Bacteria     Firmicutes       Negativicutes    Veillonellales                   Veillonellaceae           Megasphaera  micronuciformis 2.922720e-02
d5d0e236ef6bc5291a873c4a842795ac 2.400135e-03  -3.825696 Bacteria     Firmicutes          Clostridia     Clostridiales             Peptostreptococcaceae       Intestinibacter       bartlettii 3.229272e-02
884cb13b27b7dd2a3750d189c988f647 2.904909e-03   3.655812 Bacteria Proteobacteria Gammaproteobacteria  Enterobacterales                Enterobacteriaceae            Klebsiella             <NA> 3.433790e-02
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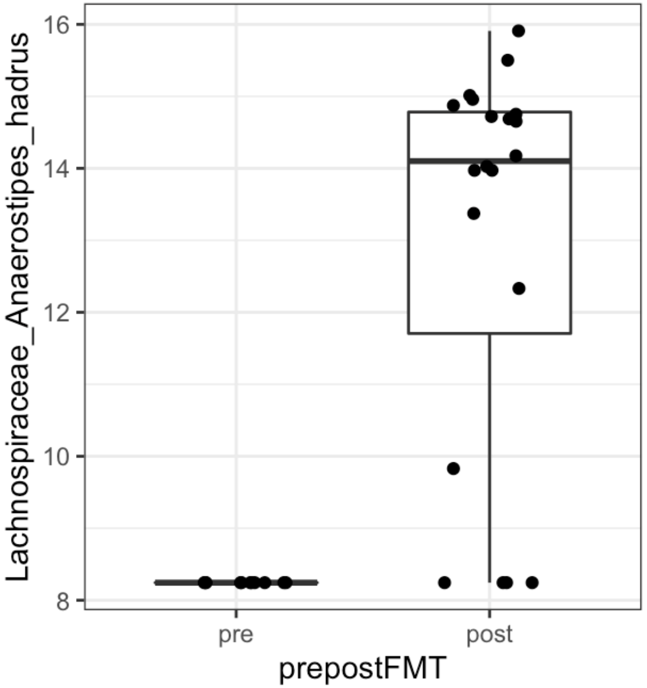
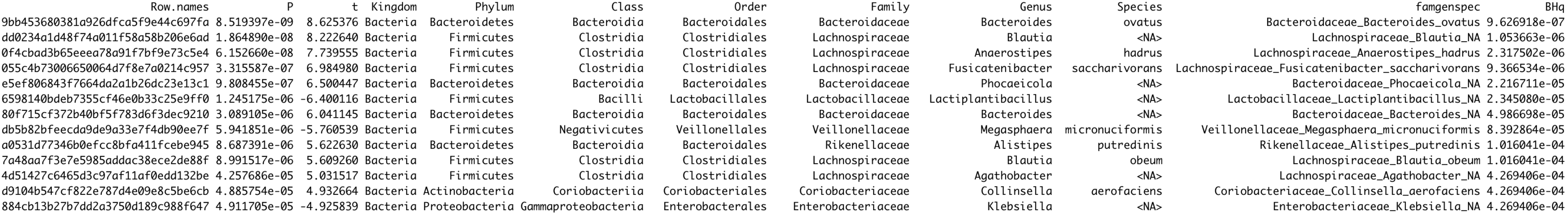


Controlling for intra-individual variance in longitudinal comparisons: linear mixed effects models

- Goal: Identify taxa that are in differential abundance between groups of samples in a longitudinal study
- Method: Control for intra-individual covariance using linear mixed effects models
  - Requires linear variables (ideally normally distributed), convert to ‘linear’-esque space using transformations
    - Ex. limma, arcsin square root, log with pseudocount
  - Perform linear mixed effects with subject ID as a ‘random effect’
  - Perform multiple comparisons correction
    - ex. Benjamini-Hochberg false discovery rate calculation (FDR ‘Q’ value)

Controlling for intra-individual variance in longitudinal comparisons: linear mixed effects models



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                       Row.names            P         t  Kingdom         Phylum               Class              Order                Family                 Genus          Species                                       famgenspec          BHq
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dd0234a1d48f74a011f58a58b206e6ad 1.864890e-08  8.222640 Bacteria     Firmicutes          Clostridia      Clostridiales       Lachnospiraceae               Blautia             <NA>                       Lachnospiraceae_Blautia_NA 1.053663e-06
0f4cbad3b65eeea78a91f7bf9e73c5e4 6.152660e-08  7.739555 Bacteria     Firmicutes          Clostridia      Clostridiales       Lachnospiraceae          Anaerostipes           hadrus              Lachnospiraceae_Anaerostipes_hadrus 2.317502e-06
055c4b73006650064d7f8e7a0214c957 3.315587e-07  6.984980 Bacteria     Firmicutes          Clostridia      Clostridiales       Lachnospiraceae      Fusicatenibacter   saccharivorans  Lachnospiraceae_Fusicatenibacter_saccharivorans 9.366534e-06
e5ef806843f7664da2a1b26dc23e13c1 9.808455e-07  6.500447 Bacteria  Bacteroidetes         Bacteroidia      Bacteroidales        Bacteroidaceae           Phocaeicola             <NA>                    Bacteroidaceae_Phocaeicola_NA 2.216711e-05
6598140bdeb7355cf46e0b33c25e9ff0 1.245175e-06 -6.400116 Bacteria     Firmicutes             Bacilli    Lactobacillales      Lactobacillaceae   Lactiplantibacillus             <NA>          Lactobacillaceae_Lactiplantibacillus_NA 2.345080e-05
80f715cf372b40bf5f783d6f3dec9210 3.089105e-06  6.041145 Bacteria  Bacteroidetes         Bacteroidia      Bacteroidales        Bacteroidaceae           Bacteroides             <NA>                    Bacteroidaceae_Bacteroides_NA 4.986698e-05
db5b82bfeecda9de9a33e7f4db90ee7f 5.941851e-06 -5.760539 Bacteria     Firmicutes       Negativicutes     Veillonellales       Veillonellaceae           Megasphaera  micronuciformis      Veillonellaceae_Megasphaera_micronuciformis 8.392864e-05
a0531d77346b0efcc8bfa411fcebe945 8.687391e-06  5.622630 Bacteria  Bacteroidetes         Bacteroidia      Bacteroidales         Rikenellaceae             Alistipes       putredinis               Rikenellaceae_Alistipes_putredinis 1.016041e-04
7a48aa7f3e7e5985addac38ece2de88f 8.991517e-06  5.609260 Bacteria     Firmicutes          Clostridia      Clostridiales       Lachnospiraceae               Blautia            obeum                    Lachnospiraceae_Blautia_obeum 1.016041e-04
4d51427c6465d3c97af11af0edd132be 4.257686e-05  5.031517 Bacteria     Firmicutes          Clostridia      Clostridiales       Lachnospiraceae          Agathobacter             <NA>                  Lachnospiraceae_Agathobacter_NA 4.269406e-04
d9104b547cf822e787d4e09e8c5be6cb 4.885754e-05  4.932664 Bacteria Actinobacteria      Coriobacteriia   Coriobacteriales     Coriobacteriaceae           Collinsella      aerofaciens        Coriobacteriaceae_Collinsella_aerofaciens 4.269406e-04
884cb13b27b7dd2a3750d189c988f647 4.911705e-05 -4.925839 Bacteria Proteobacteria Gammaproteobacteria   Enterobacterales    Enterobacteriaceae            Klebsiella             <NA>                 Enterobacteriaceae_Klebsiella_NA 4.269406e-04
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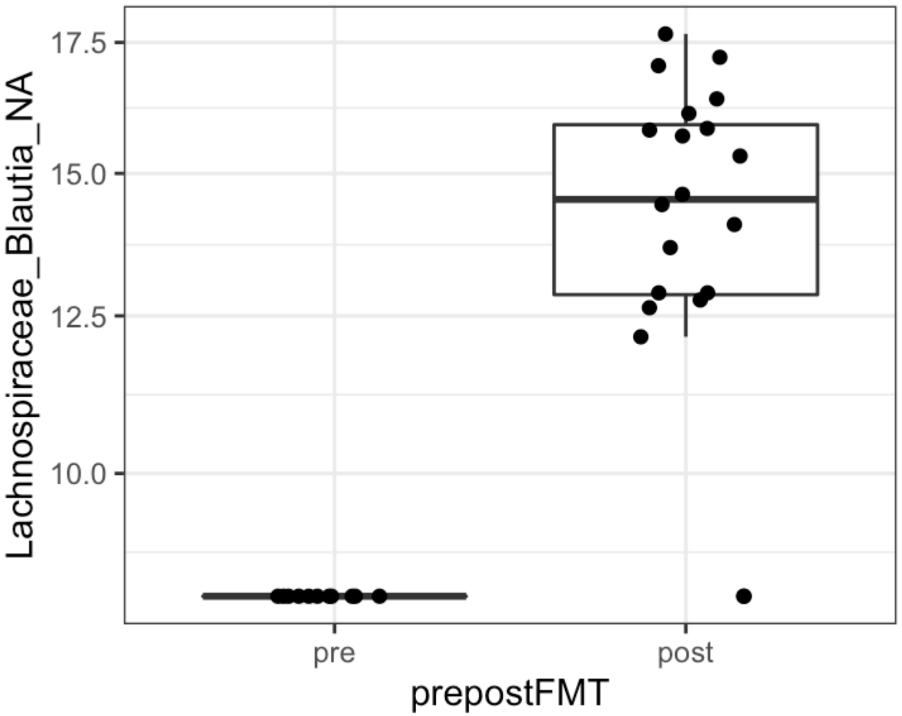
Lachnospiraceae_Blautia_NA
pre
post
prepostFMT