Characterization of the changes in the microbial diversity and microbiome expression in the upper respiratory track of ferrets (*Mustela putorius furo*) during influenza virus infection

Mount Sinai School of Medicine:

Adolfo Garcia-Sastre

Rafael Medina

Nacho Mena

U Pittsburgh School of Medicine:

Elodie Ghedin

JCVI:

Monika Bihan

Kelvin Li

Barbara Methé

Update for GSC Meeting 07/19/12

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Project Questions

- Following Influenza viral infection of the host (2009 H1N1 virus) is the bacterial community structure affected?

- If so, determine the changes that microbial communities undergo over the course of an influenza virus infection

- Identify specific microbial populations that might be altered during influenza infection

Phase I

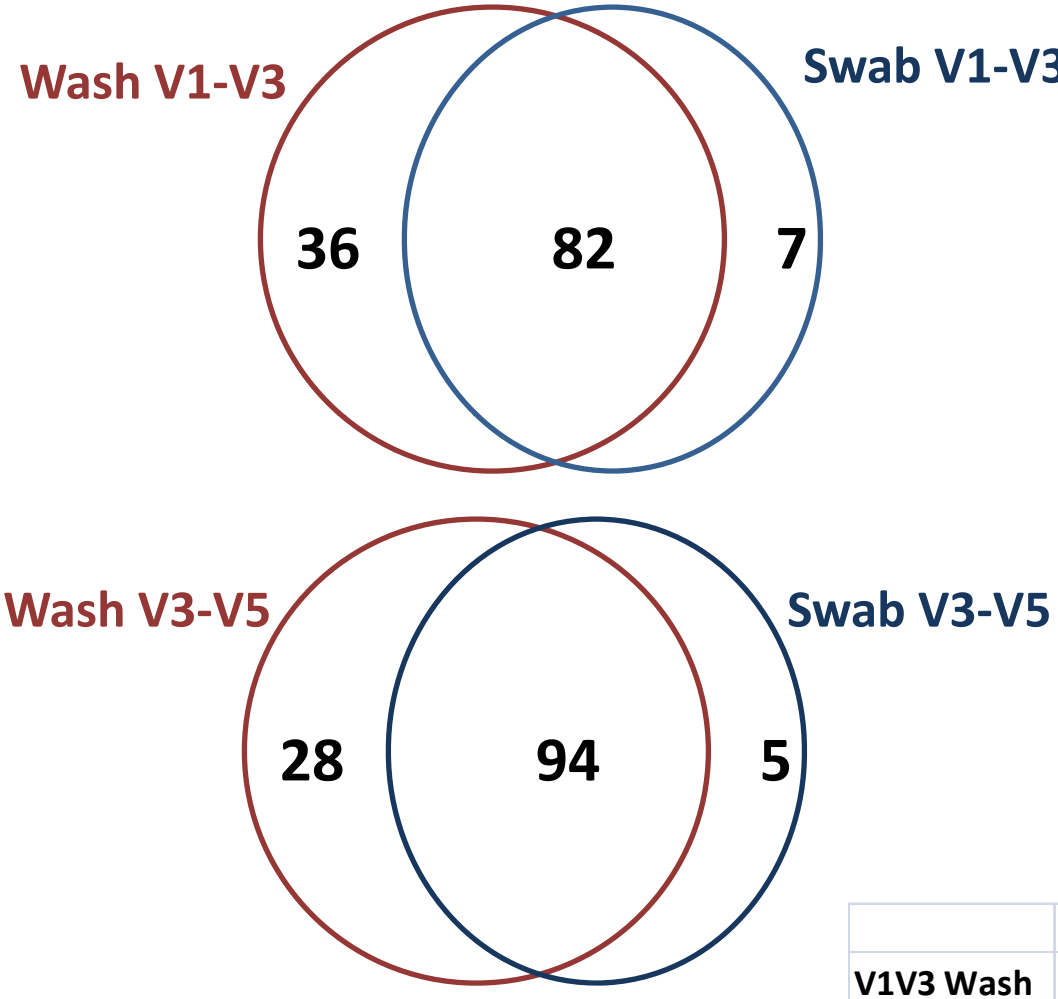
- 2 uninfected
  - Nasal swab versus nasal wash
  - Day 0 and Day 2
  - Total specimens: 8
- 2 infected + 1 uninfected control
  - Nasal washes only
  - Day 0, Day 2, Day 3
  - Total specimens: 9

Pilot Experiment (Phase I)

- Total of 17 specimens
  - –3 technical replicates of 16S amplicons
  - –2 regions for each: V1-V3 and V3-V5
  - –For each round of PCR, 1 positive and 1 negative control

- Sequencing: average of 4175 classifiable reads per sample (post processing for short reads, low quality reads, chimeras etc)

More genera were recovered and generally higher diversity in nasal washes versus swabs



	Shannon	Tail	Discovered Genera
V1V3 Wash	2.59	11.41	118
V3V5 Wash	2.40	10.20	122
V1V3 Swab	2.32	8.85	89
V3V5 Swab	2.48	9.08	99

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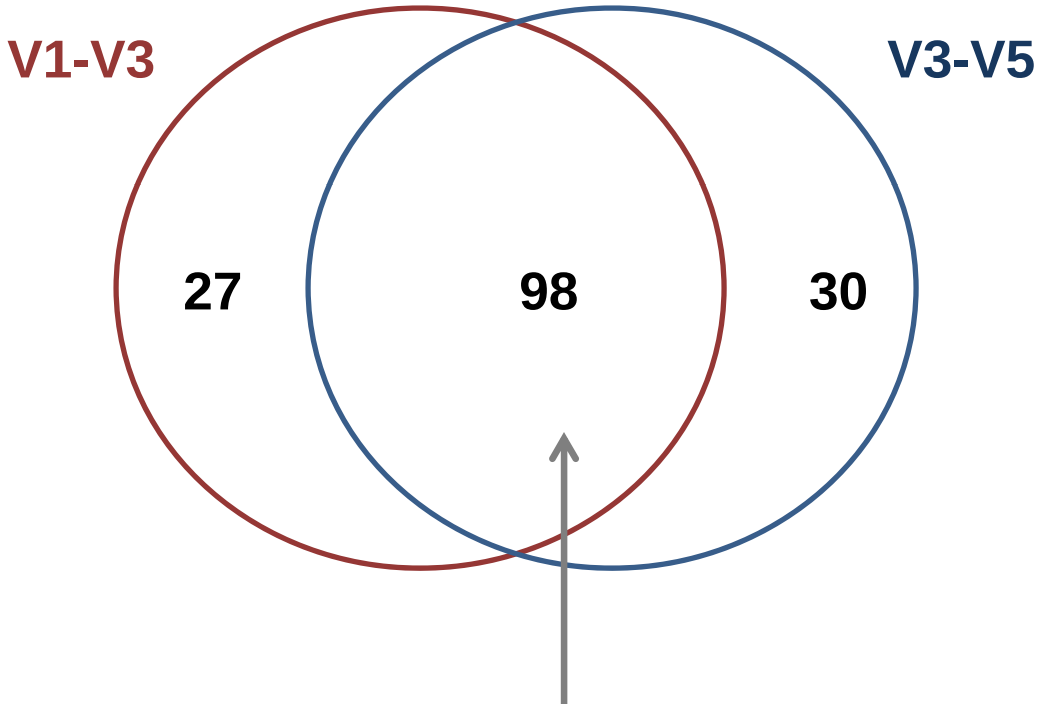
Similar taxonomic profiles were recovered using different variable regions of the 16S rRNA gene



Shared genera represents the majority of the total abundance

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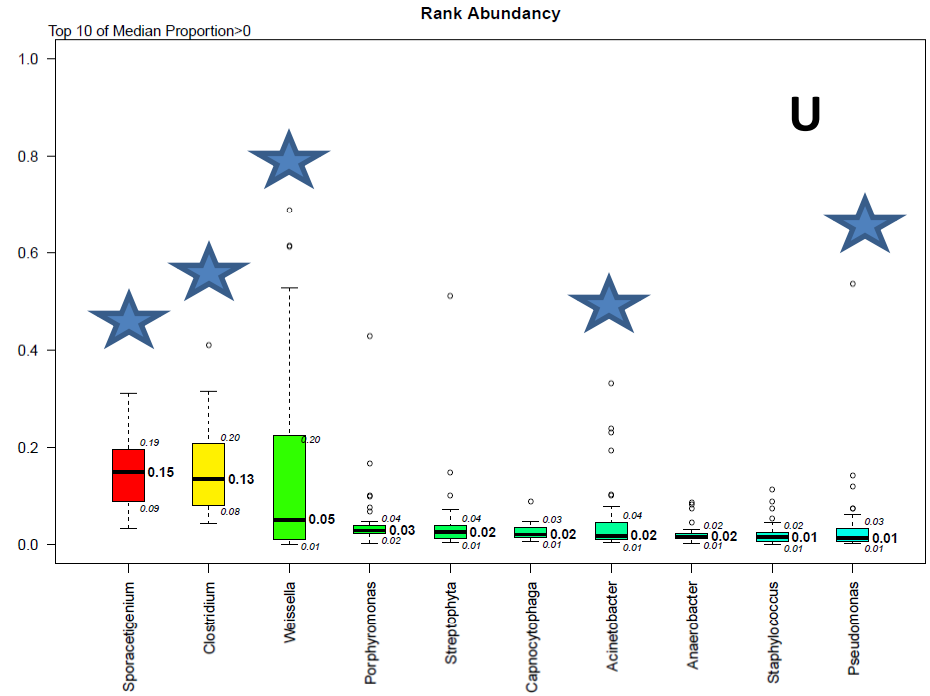
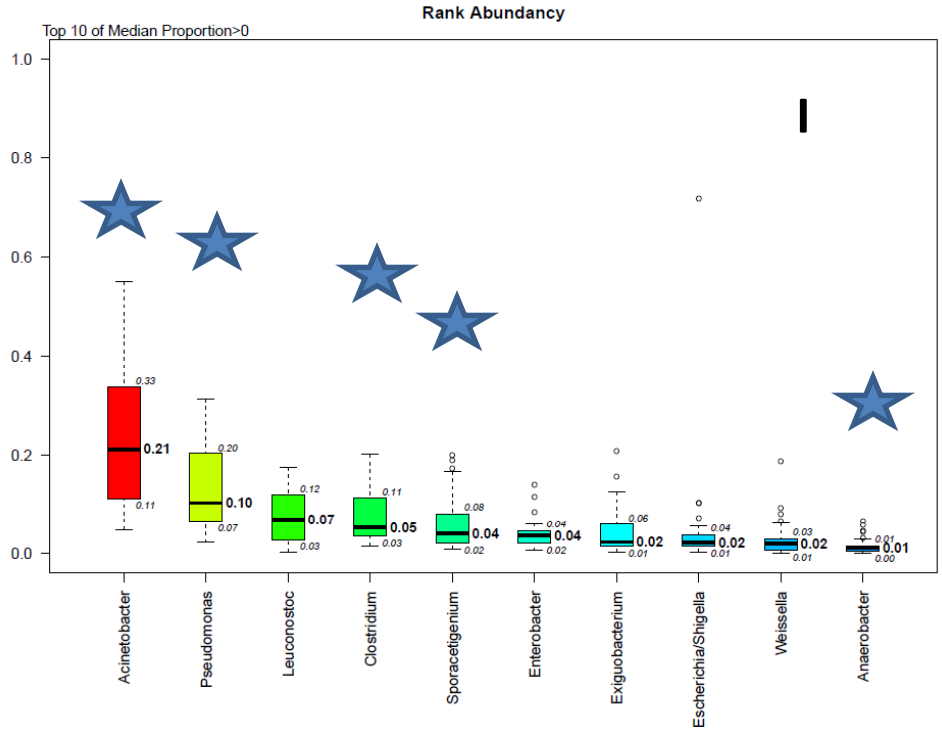
Phase I Summary

- Higher diversity in nasal washes
- Similar genera recovered from two portions of the 16S rRNA gene, especially in the high abundant genera (but not the same)
- Typically the V1-V3 primer set “behaves” better in the lab

Phase II

- 7 Infected ferrets (H1N1 2009)
- 7 Uninfected ferrets (controls)
- Nasal wash collections
- Time course: Day 0, 1, 3, 5, 7, 14
- Sequenced the 16S rRNA gene in the V1-V3 and V3-V5 regions
- Analysis of this data is currently in progress, the remainder of the slides in this presentation will share some highlights of this ongoing investigation





Box and Whisker Plots of Rank Abundancy- top 10 genera from all Uninfected (U) vs. Infected (I) samples

Five of the top 10 genera are the same between the U and I groups (indicated with blue stars)

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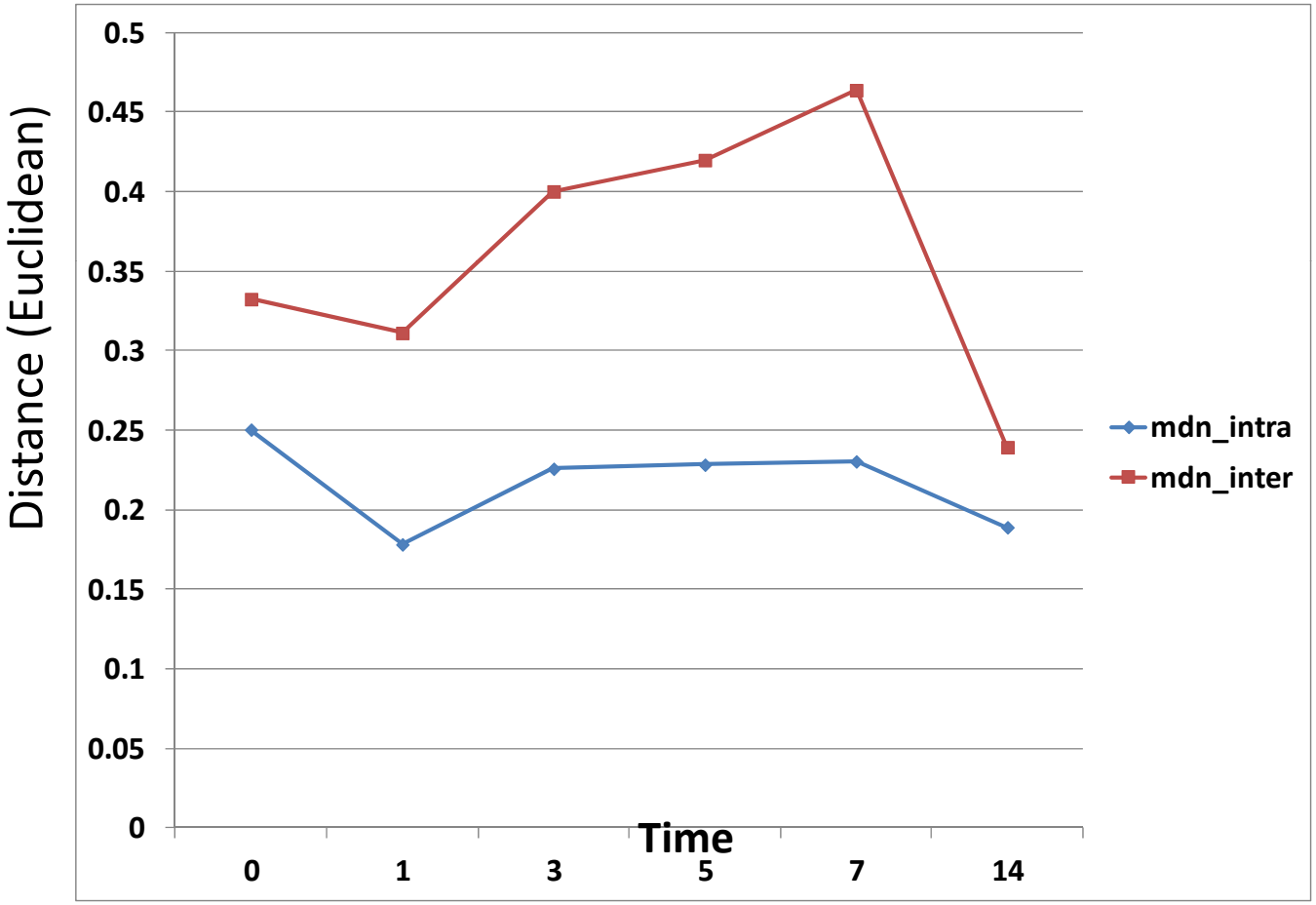
Phase II

Comparing Infected vs. Uninfected intra-cluster to inter-cluster distances

(All p-values for inter-cluster distances <0.01 based on Wilcoxon Rank Sum Test)



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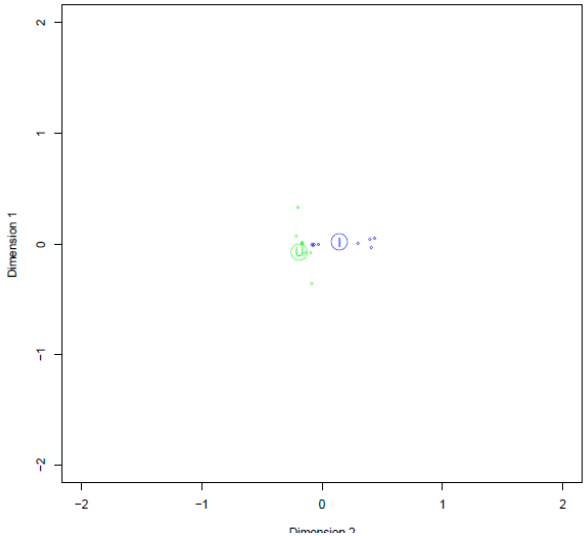
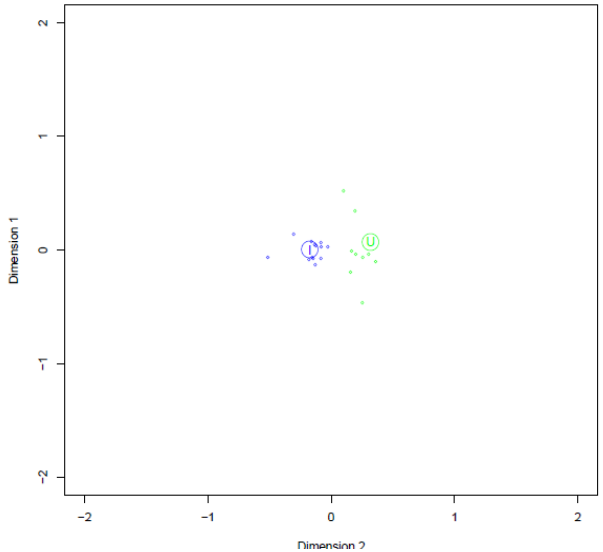
MDS Plots U vs I

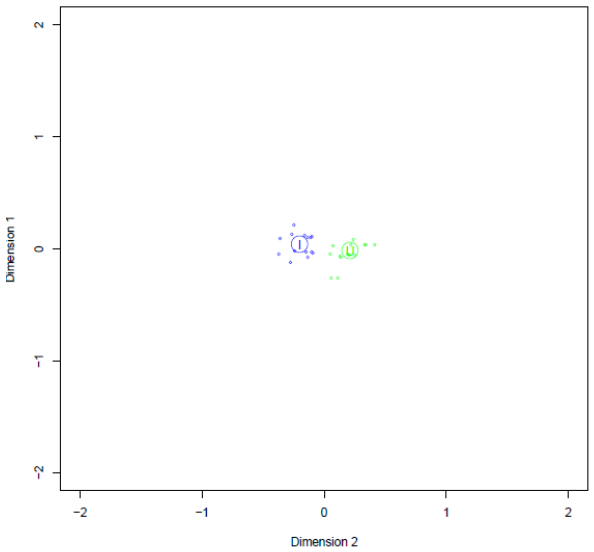
U Centroid, Uninfected I Centroid, Infected



Day 0



Day 1



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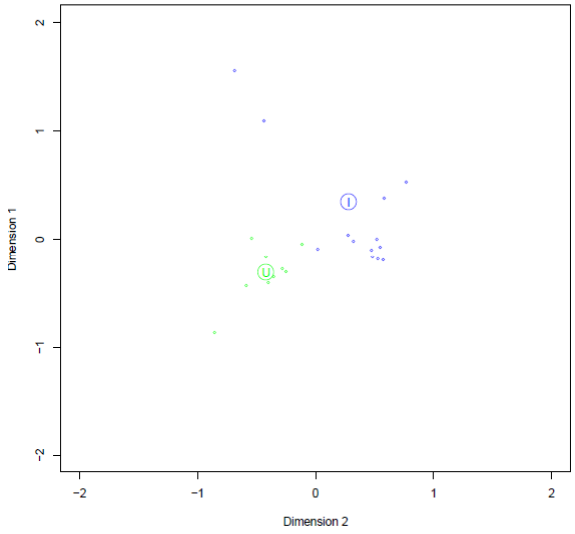

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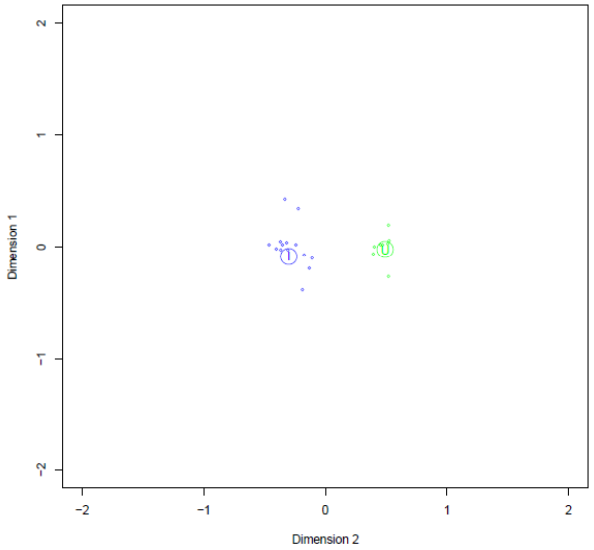
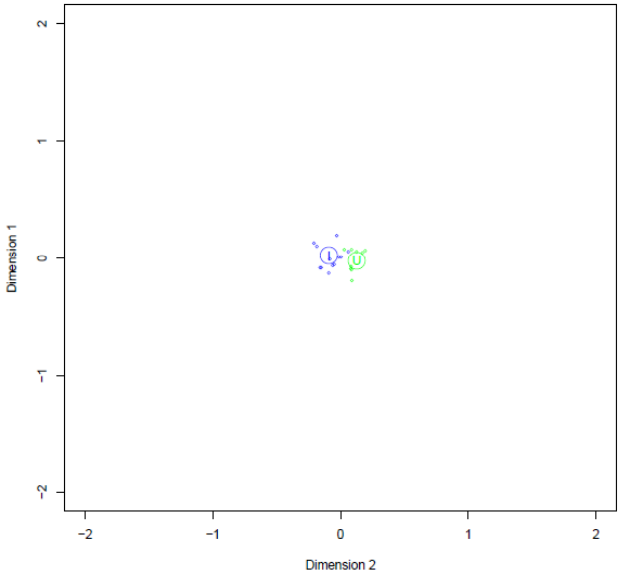


Day 7



Day 14

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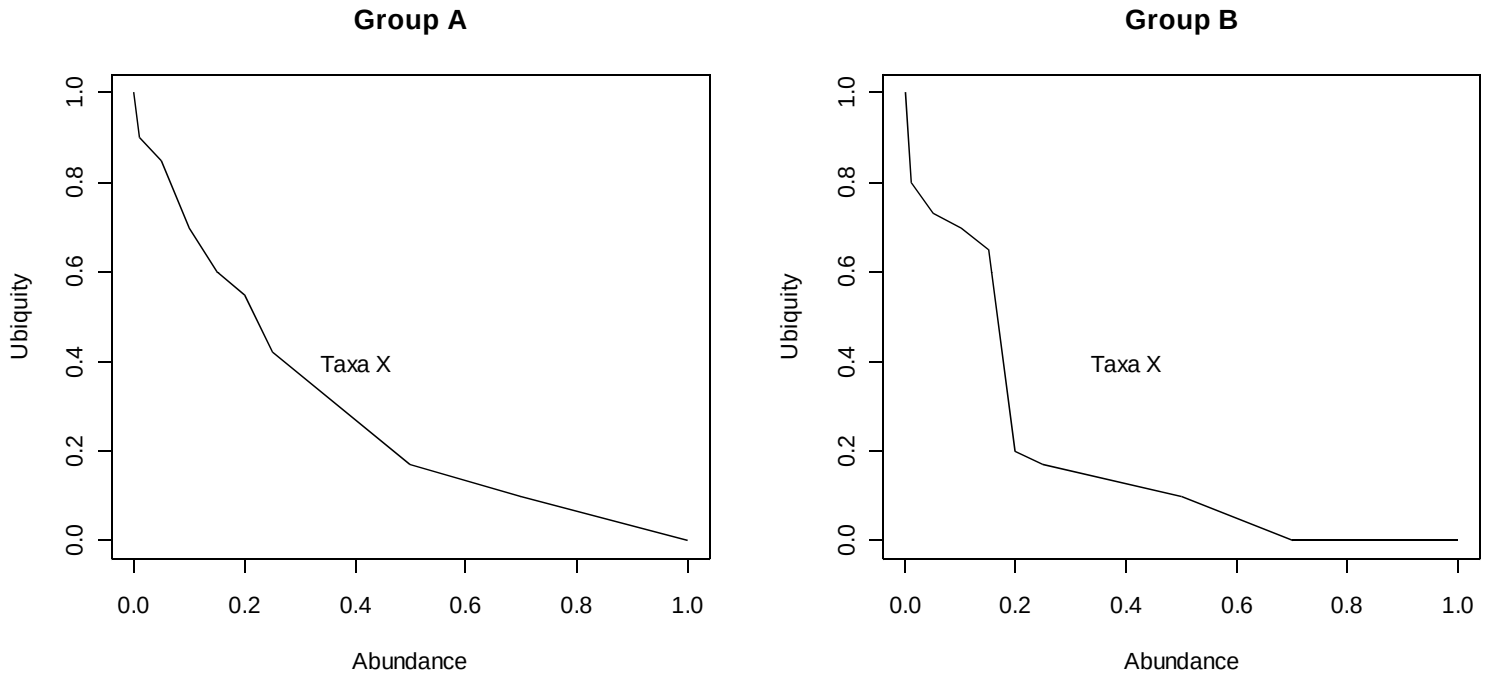
Microbiome Analysis

- One concept we wish to study is the nature and extent of microbial diversity across individual ferrets in the treatment and control groups over time
- To do this we have need to examine both relative abundance and ubiquity of taxa (typically at the genus and OTU level)
- A set of taxa can be identified quantitatively by defining a threshold for ubiquity and abundance
  - Ubiquity: If 6 out of 10 donors have a taxa of interest in their microbiome, the ubiquity of that taxa is 60%
  - Abundance: If 15% of the reads recovered from a donor's sample are identified as taxa X, the abundance of X in the sample is 15%

Comparing Ubiquity vs. Abundance



- We want to compare Group A versus Group B for the same Taxa X

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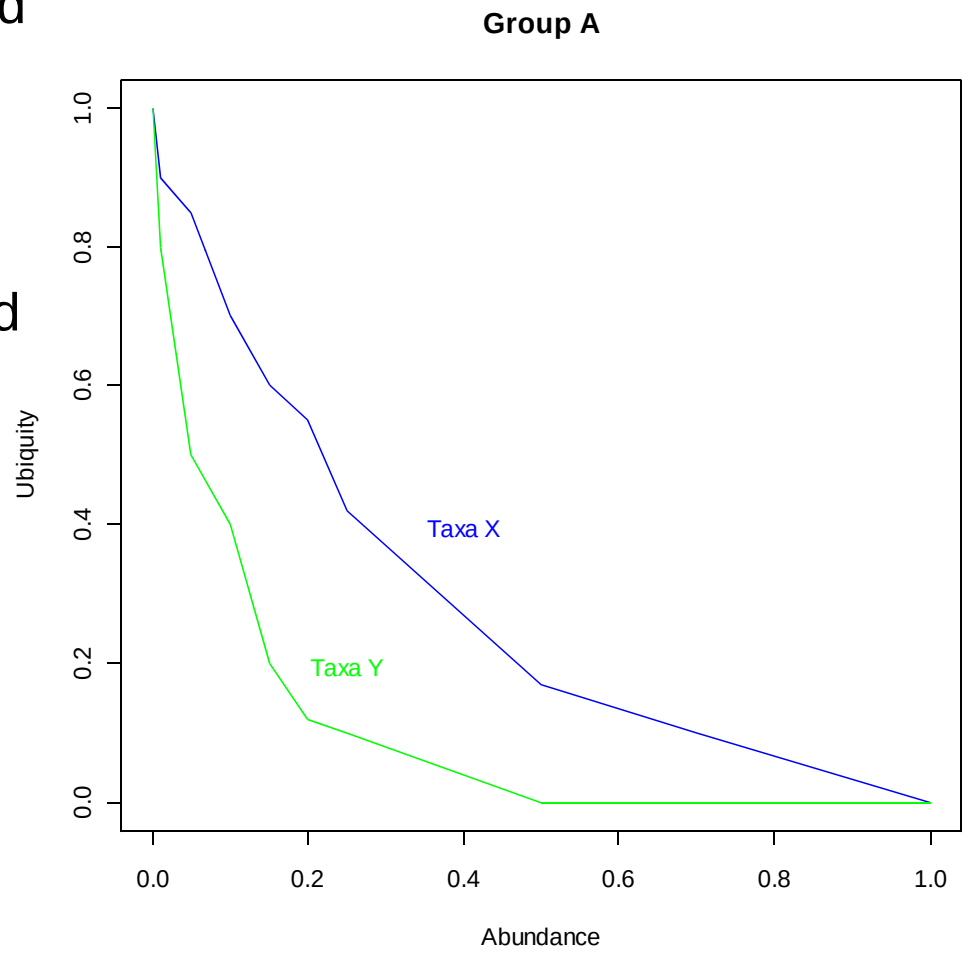
Multiple Taxa

- Multiple taxa can be compared simultaneously
- Taxa with lower curve is less prevalent in group
- Taxa X is more ubiquitous and abundant than Taxa Y
- Sometimes lines can cross, suggesting additional complexity



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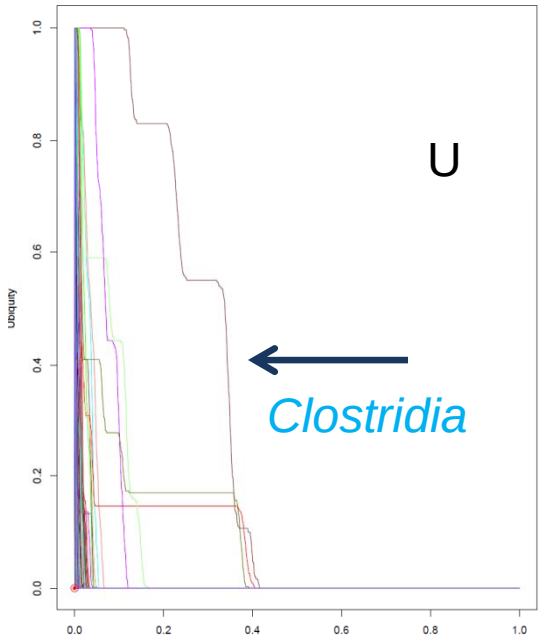
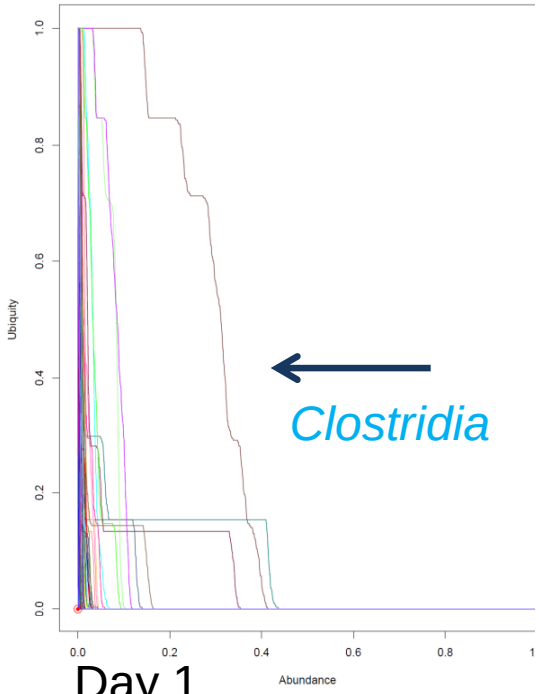
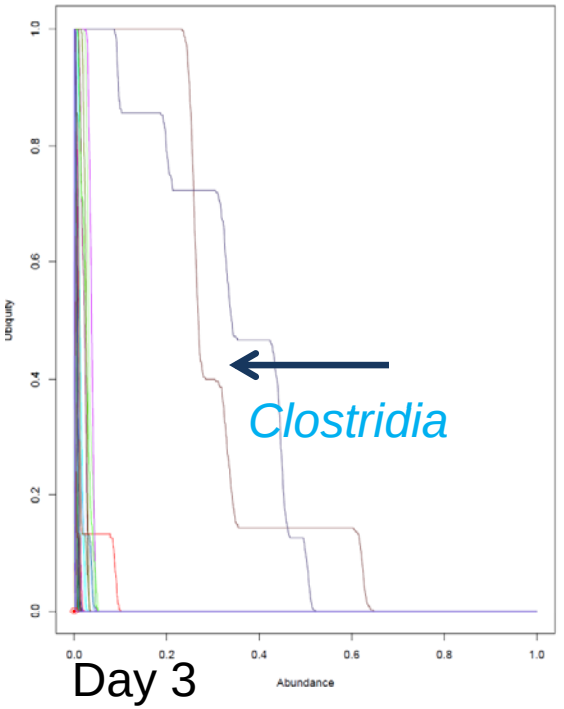


Day 0



Day 1



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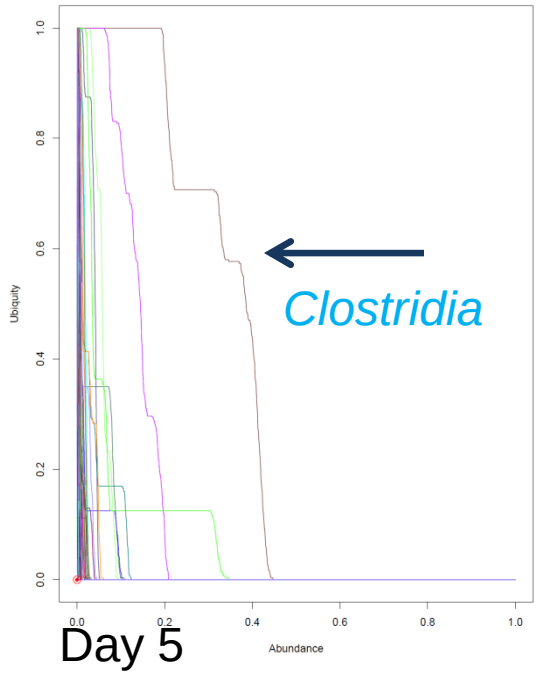

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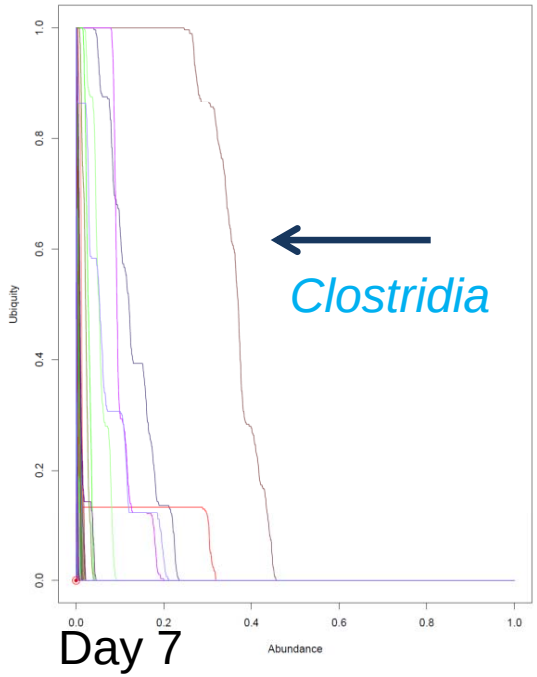

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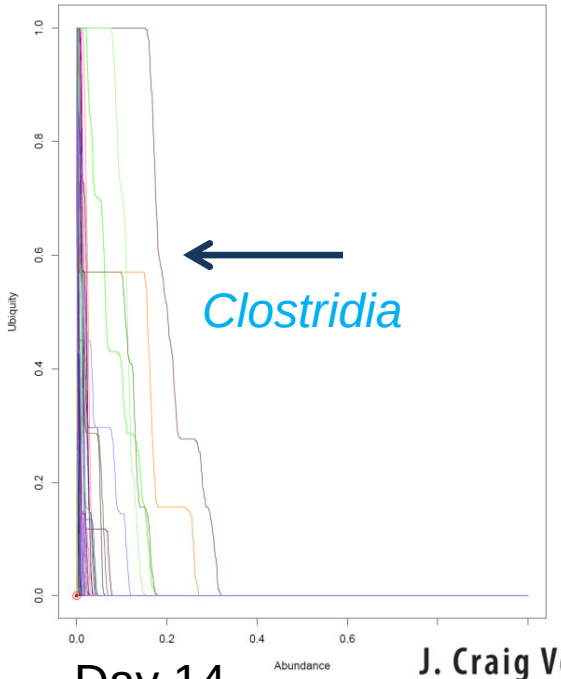

Day 14

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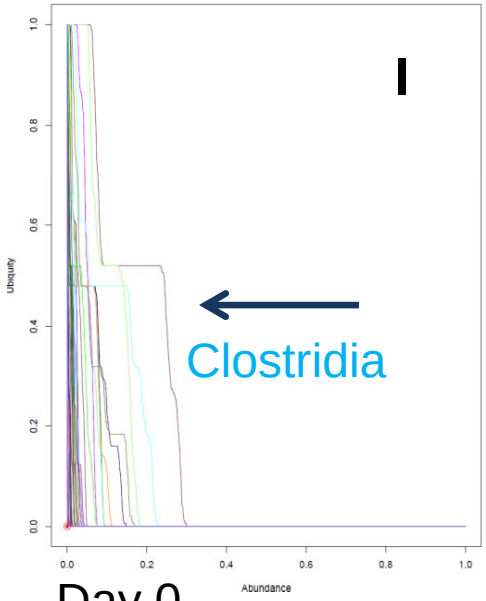

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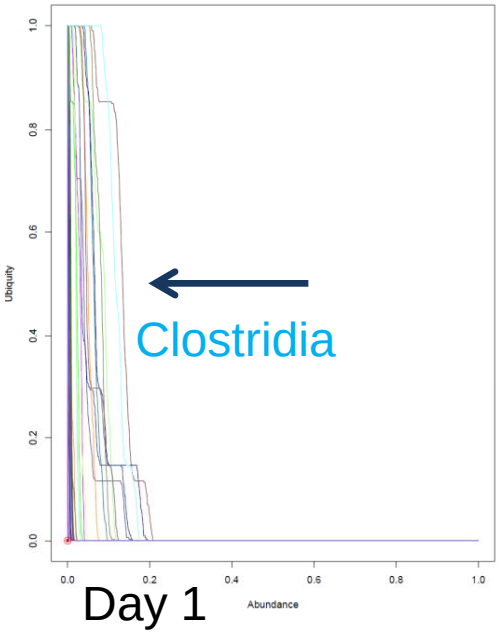
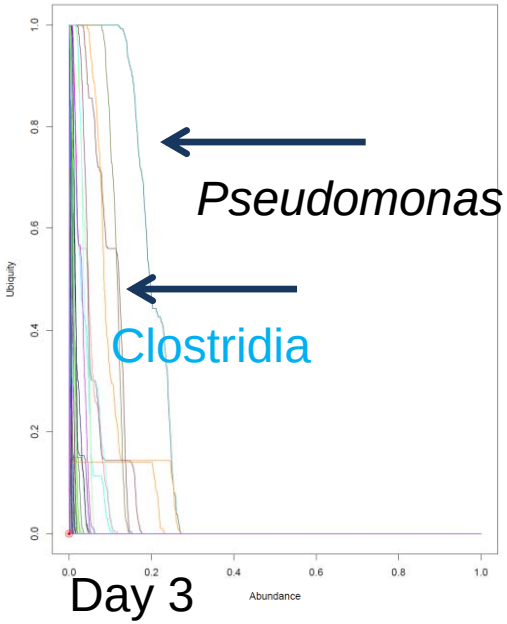


Day 1



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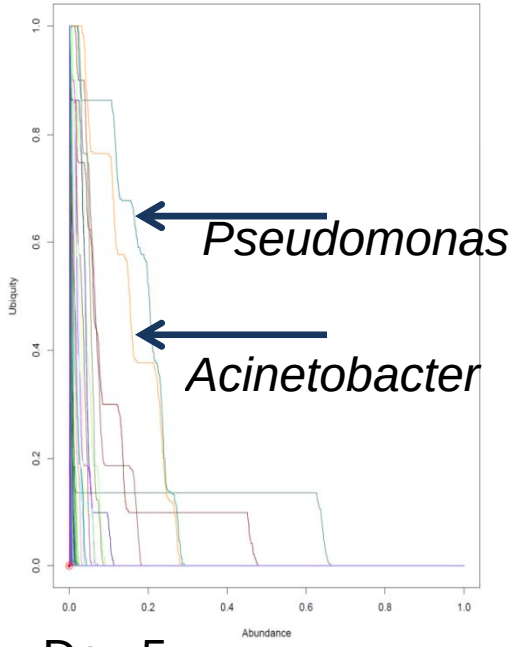

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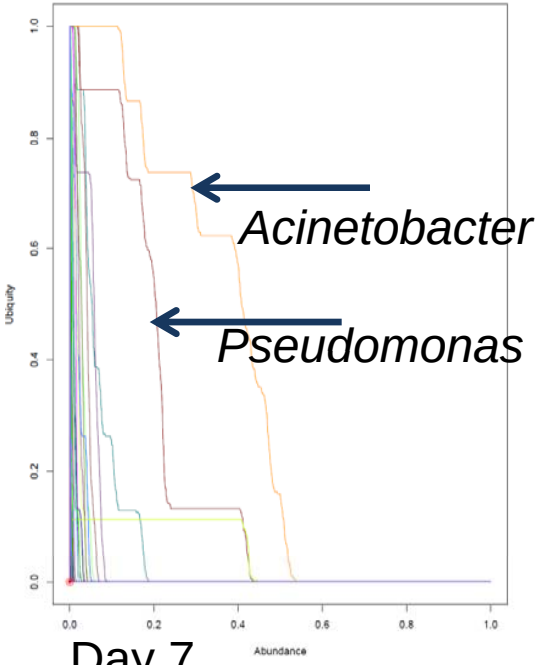

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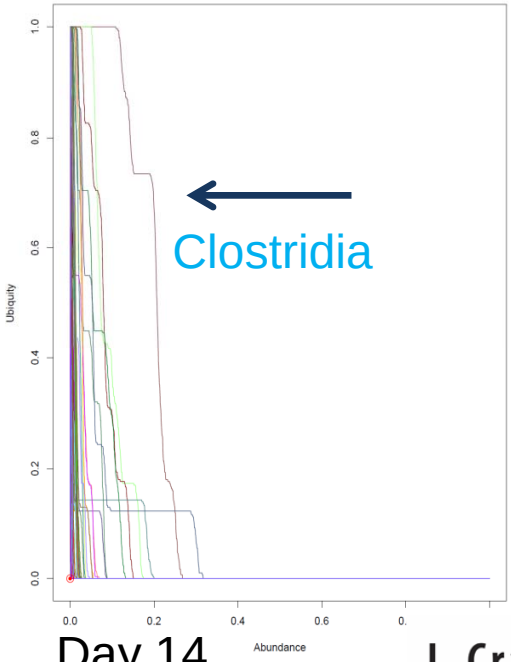

Day 14

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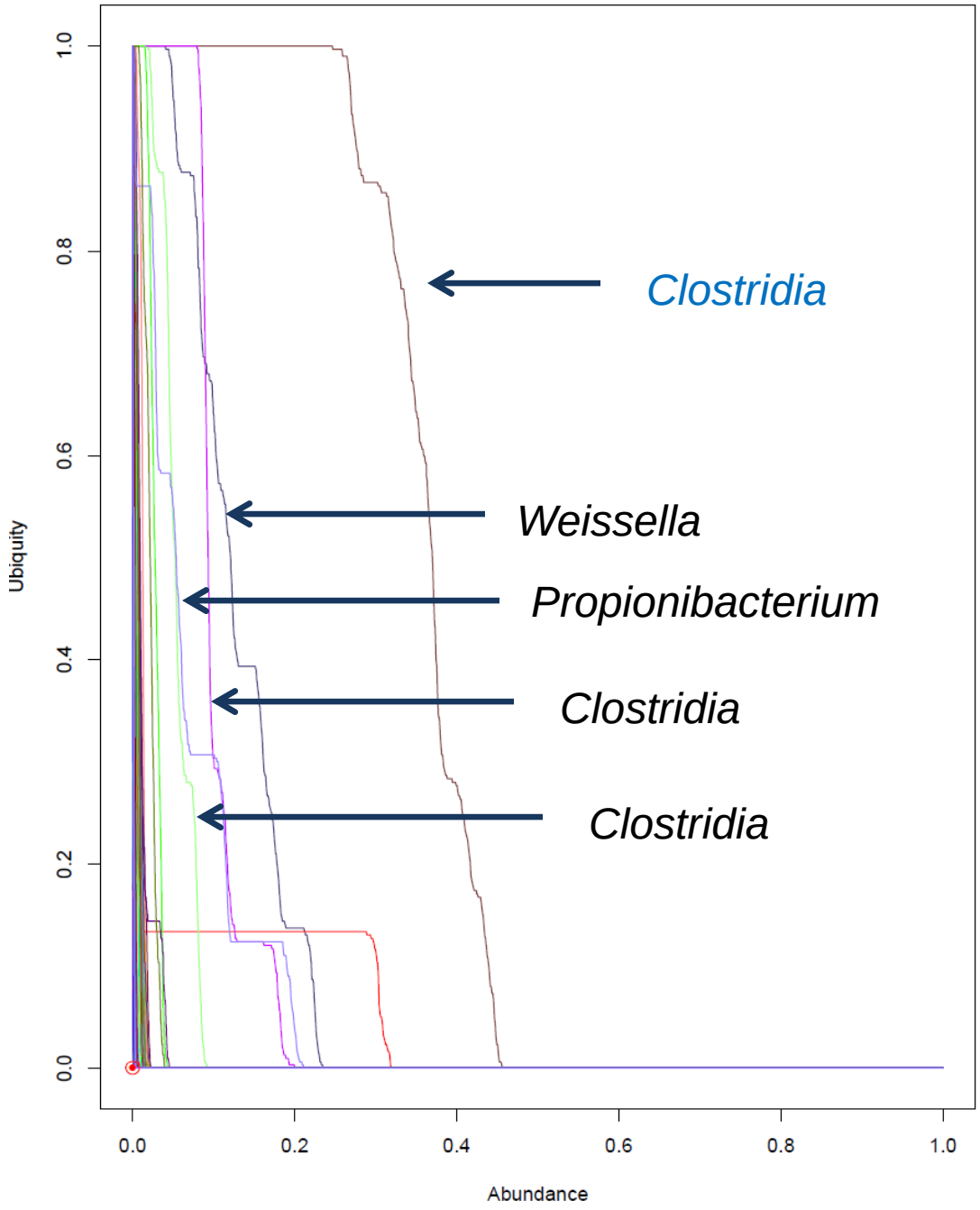

Ubiquity-Abundance Plot
Unifected Day 7

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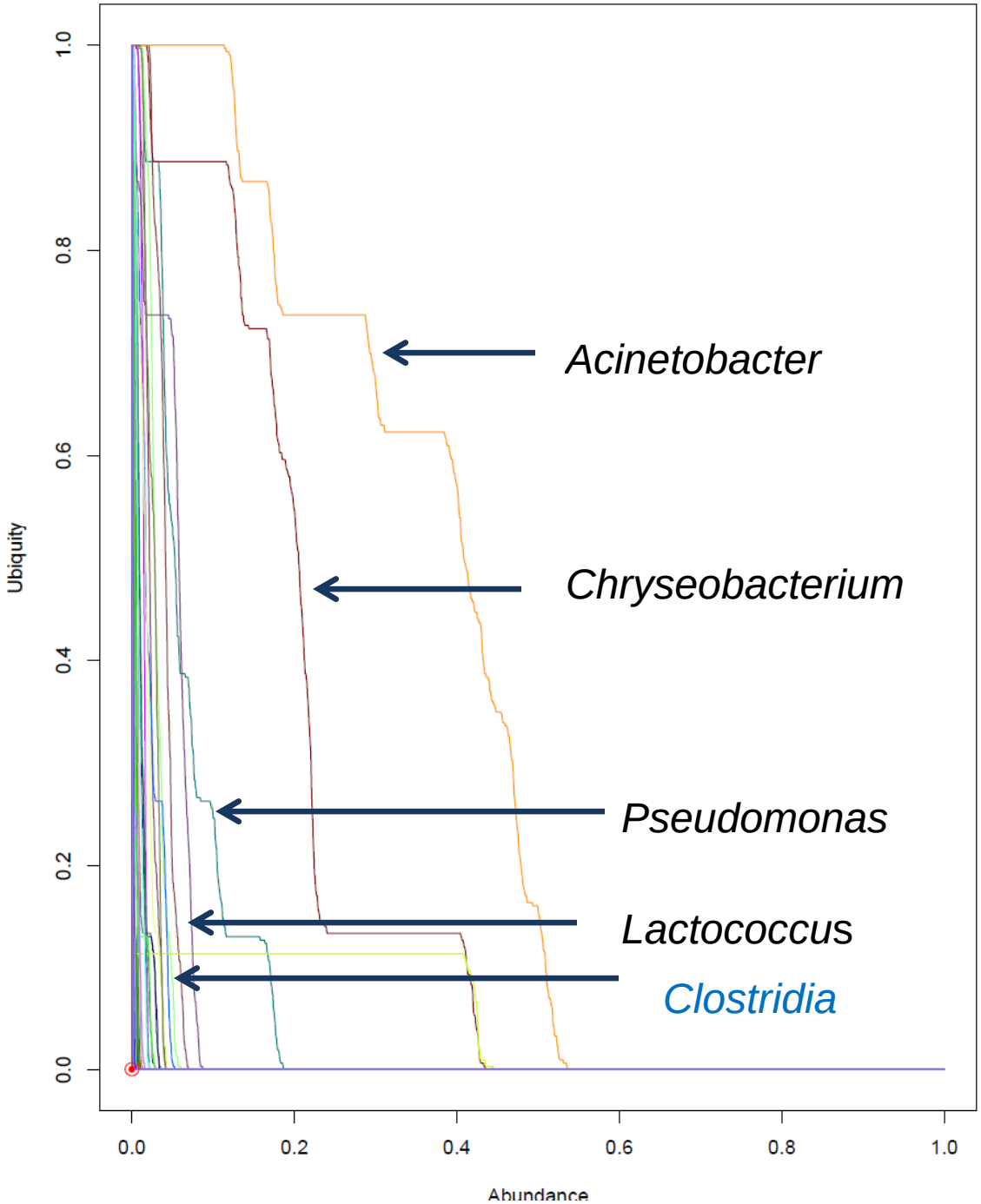

Ubiquity-Abundance Plot
Infected, Day 7

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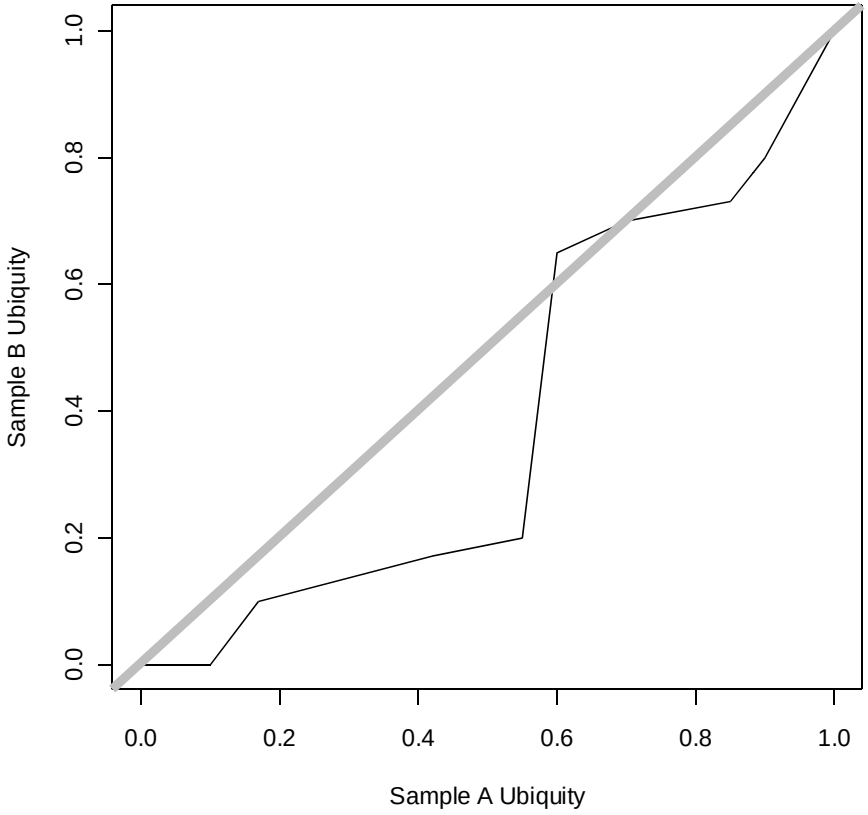
Comparing Microbial Communities

- When a treatment is applied to a group of individuals:
  - Individuals will not respond identically
  - Microbial taxa will not respond identically
  - Curves will not just shift up/down left/right
- Need to consider shifts of ubiquity across all abundances

Ubiquity-Ubiquity (U-U) Plot

- Akin to quantile-quantile plots used in statistics as a graphical method for examining the distributions of two data sets
- Substituting abundance for quantiles
- When ubiquity's match between groups, they will fall on the gray line
- The further the U-U line deviates from the gray reference, the more the taxa has changed between groups.
- Deviation below the gray implies greater general abundance of a taxa in group A compared to group B



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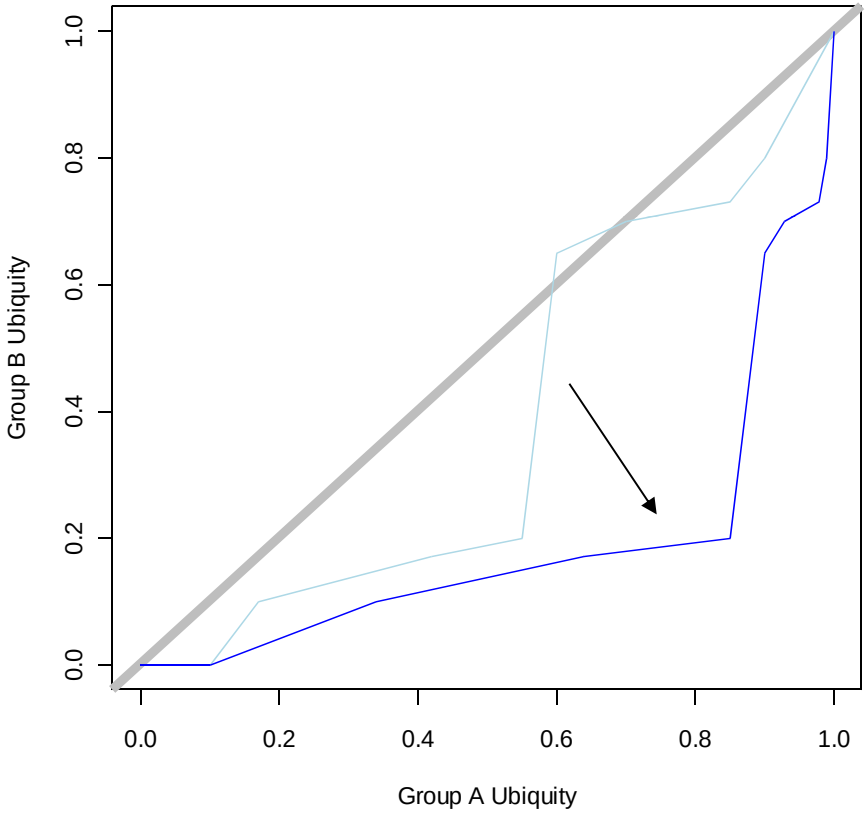
Visualizing Microbiome Shifts

- The taxa indicated by the arrow has increased abundance across all group members
- This is apparent because the U-U line has been pulled further away from the gray reference line



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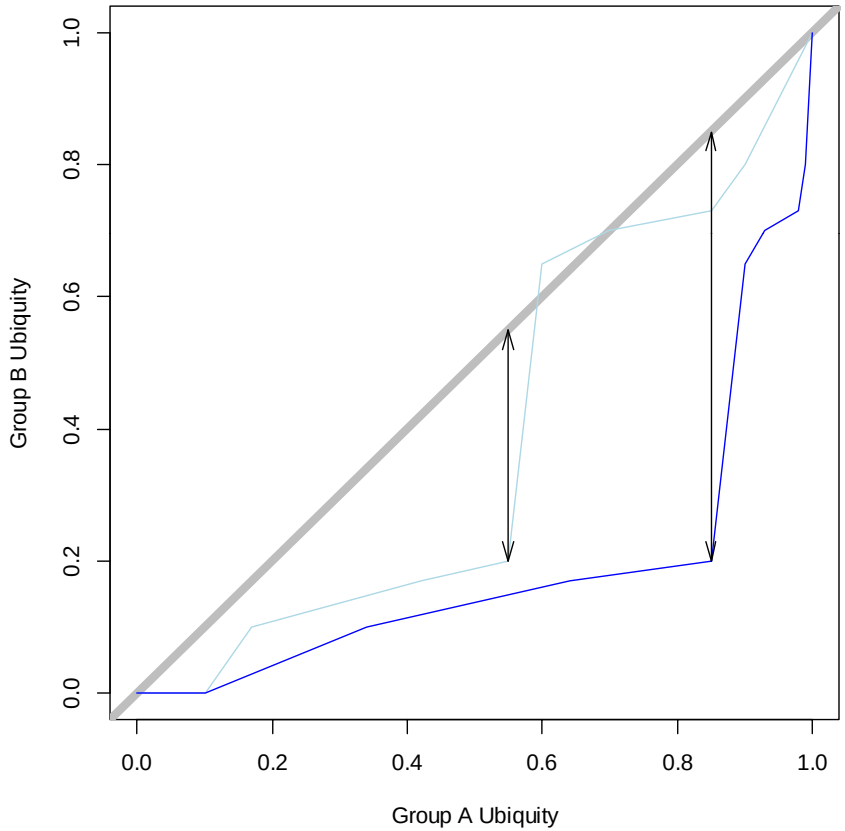
Measuring the Shift

- The K-S statistic for a single taxa between two groups is the maximum difference between the two U-U lines
- max(abs(diff(Ubiquities(A)-Ubiquities(B))))
- The max distance between the U-U line and the grey reference line
- The black arrows represent the magnitude of the K-S statistic
- As the dark blue lines move further away from the reference gray line, the, K-S increases



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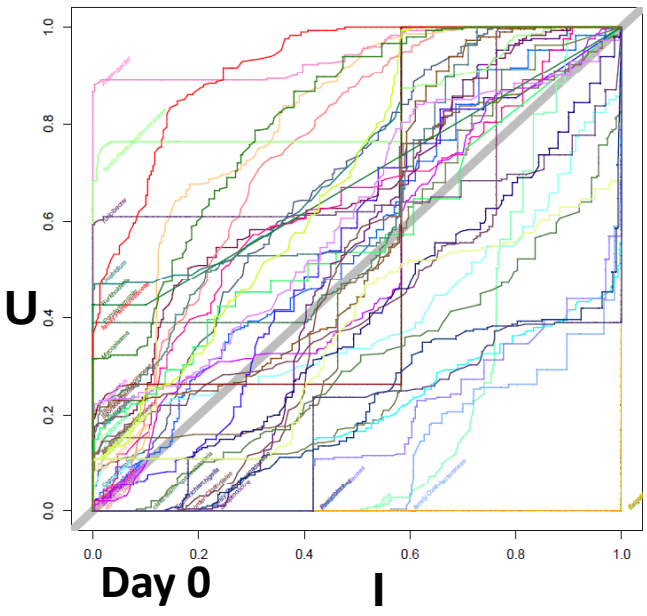
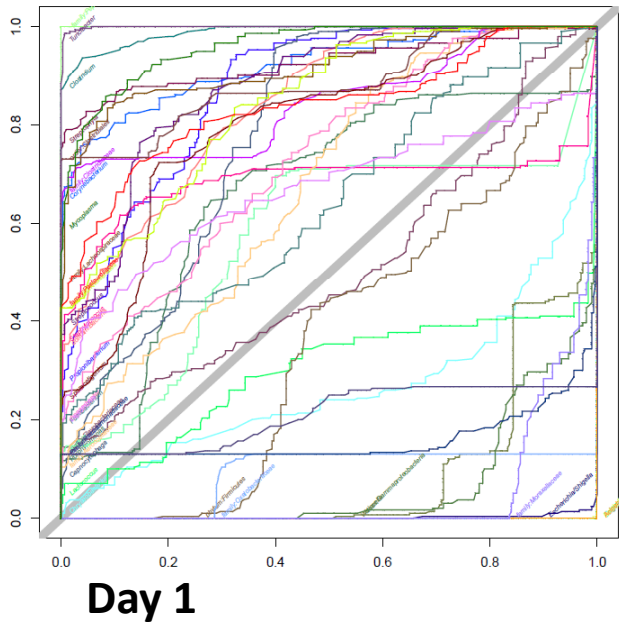
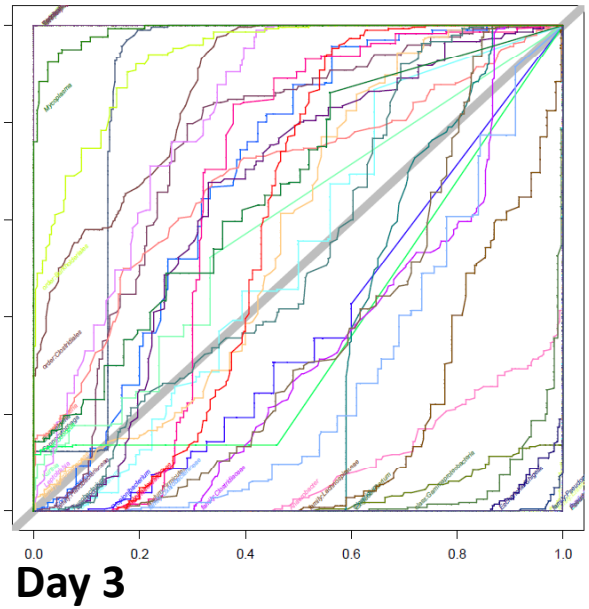
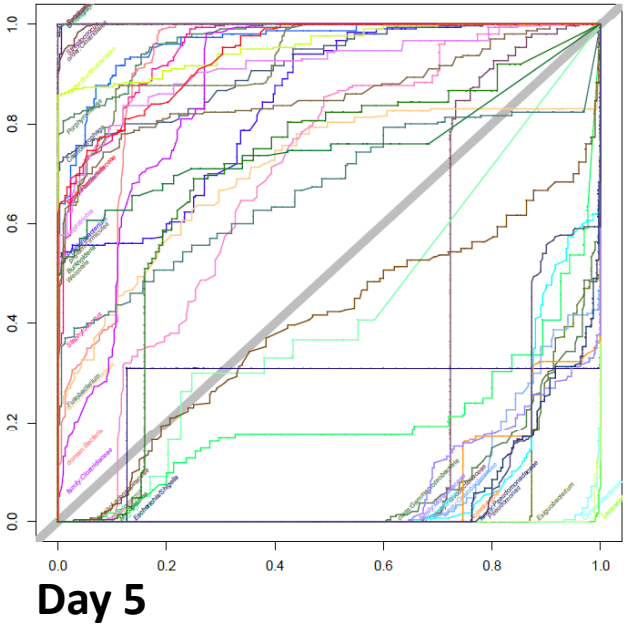
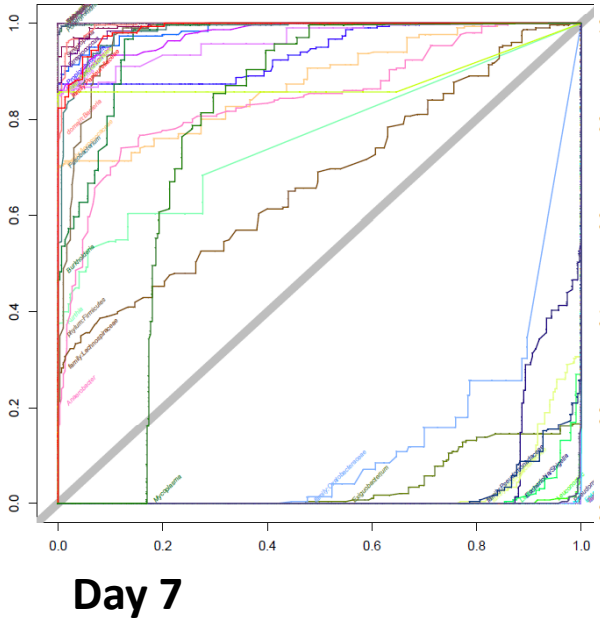
Ubiquity-Ubiquity Plots of Uninfected (Y-axis) vs. Infected (X-axis) - Genus level

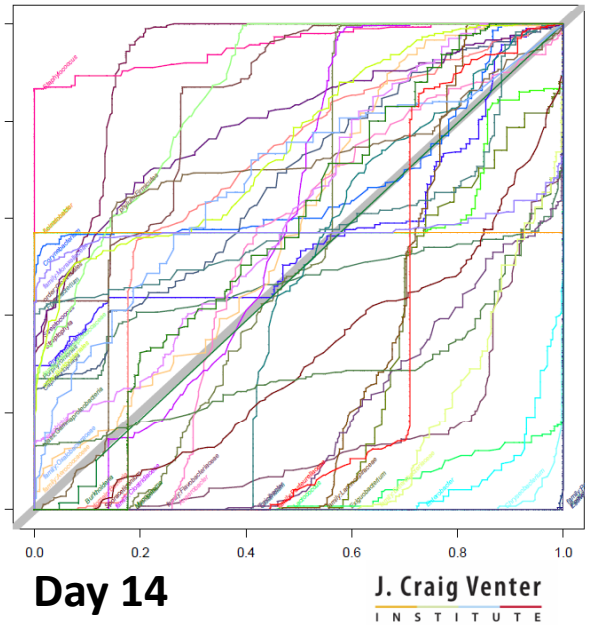












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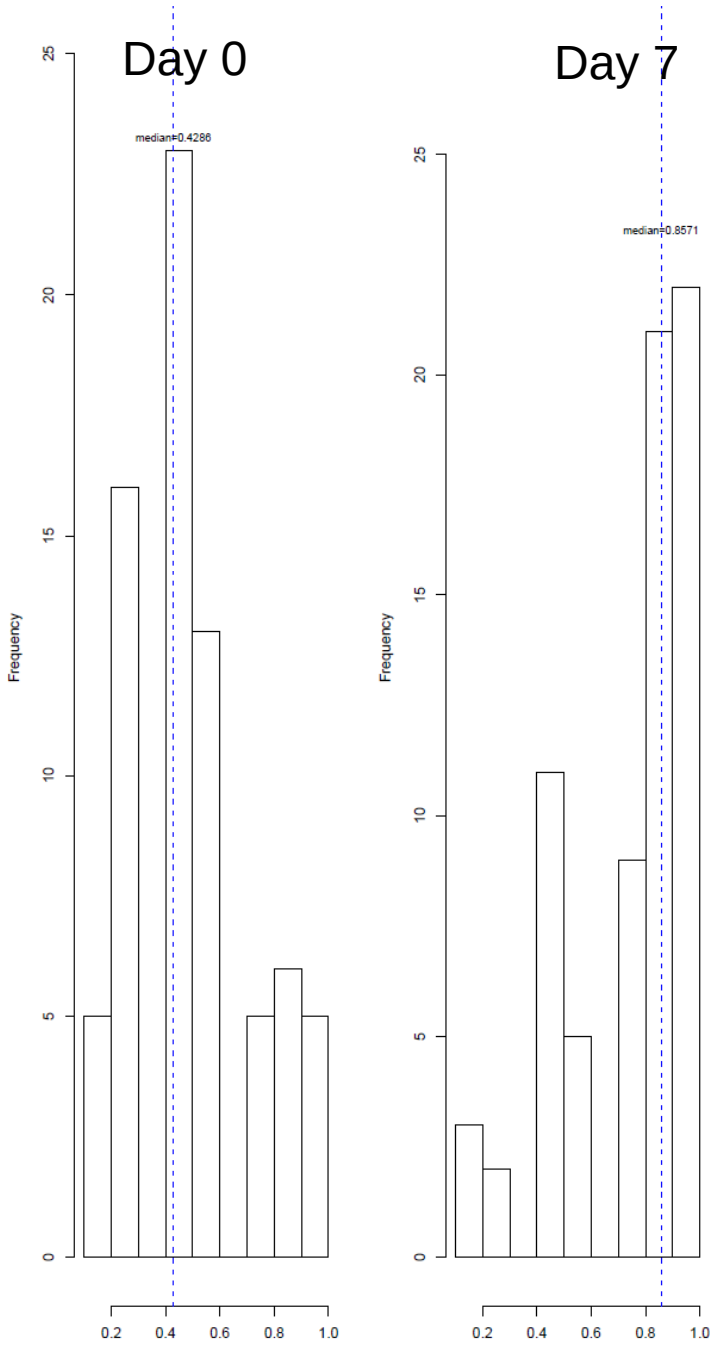
Measuring Differences between Time

- Quantify differences between infected and uninfected groups in the same time period
  - For each pair of groups (infected and uninfected) at each time point, generate a set of K-S statistics, one for each taxa
- To compare time periods, compare the median K-S statistic using the nonparametric Wilcoxon Rank Sum Test
  - If the median K-S statistic is statistically different between time periods, then the treatment may have had an effect
  - Note: The increase of one taxa's proportion necessarily reduces the proportion of another taxa's proportion. This makes all the K-S not strictly independent, so the actual p-values may be larger than computed.



Example using the Wilcoxon Rank Sum Test (WRST) to compare time periods- Day 0 vs. Day 7

Wilcoxon Rank Sum Test:
p-value = 0.0000
Left Median = 0.4286
Right Median = 0.8571

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Comparison of p-values from WRST of Infected and Uninfected Groups by Time

	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
Day 0	-	0.0007	0.0002	0.0001	0.0000	0.0671
Day 1		--	0.648	0.6101	0.005	0.0737
Day 3			--	0.9731	0.017	0.0233
Day 5				--	0.009	0.0141
Day 7					--	0.0000
Day 14						--

All time points compared to Day 0 differ significantly
Fewer differences between Days 1,3,5

Summary

- Observed differences in microbial community diversity in the URT of ferrets infected with influenza compared to untreated controls
- These differences appear greatest at Day 7 of infection
- Shifts between infected and uninfected microbial communities are seen in both relative abundance and ubiquity of taxa at the genera and OTU levels
- Based on initial comparisons, among the changes observed is increased ubiquity and abundance of microorganisms related to *Acinetobacter* and *Pseudomonas* in the infected group
- Next steps in the project include additional analysis of 16S profiles and generation of transcriptomic data from selected samples