Fine-tuning the association between pediatric arthritis and *Faecalibacterium prausnitzii*

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Wonderful World of Technology

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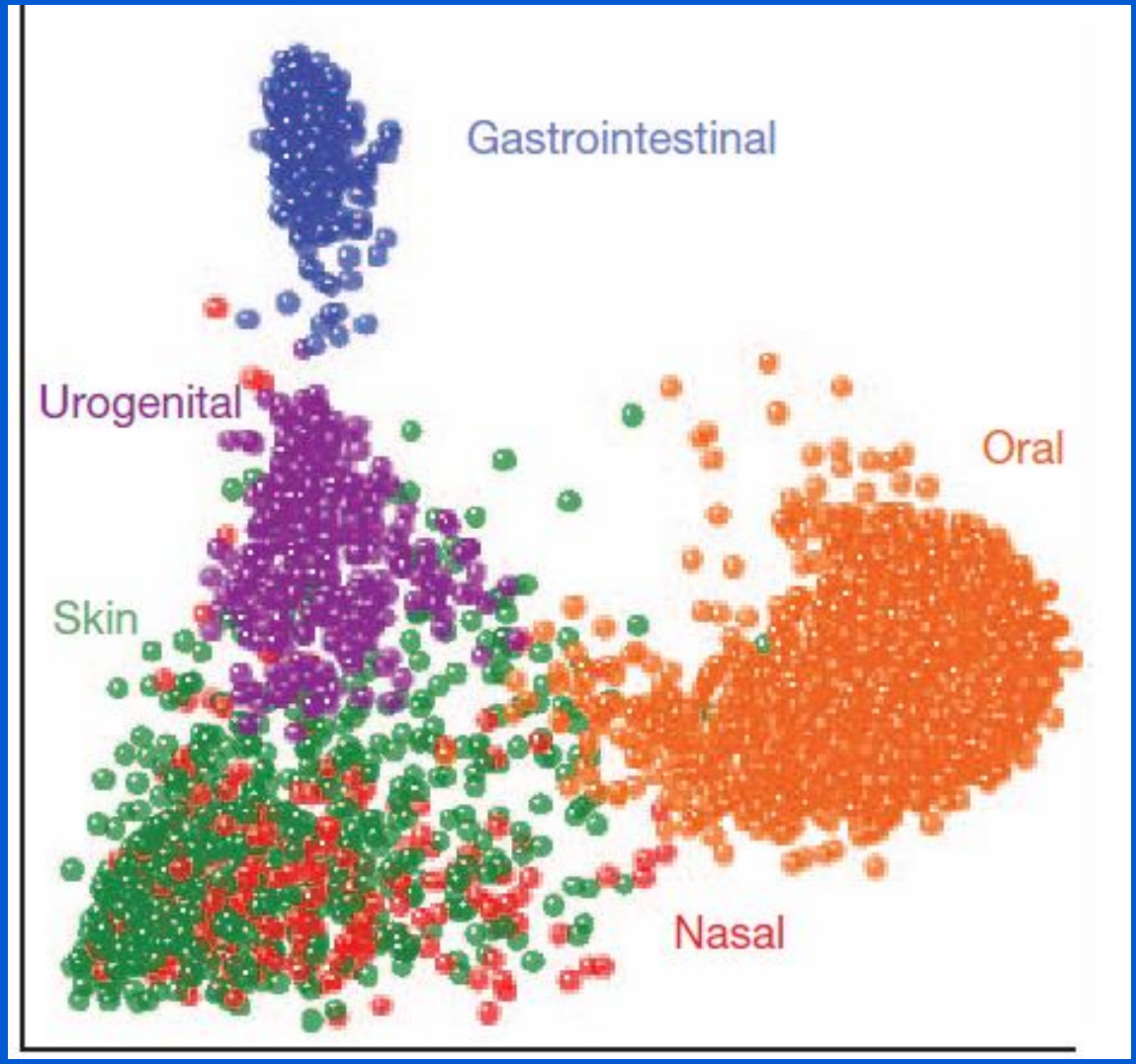
Microbiota and human diseases

- Inflammatory bowel disease
- Arthritis
  - Juvenile idiopathic arthritis
  - Rheumatoid arthritis
  - Spondyloarthritis
- Type I diabetes
- Obesity / metabolic syndrome

Outline

- **Where to sample**
- **Sequencing**
- **Data analysis**
- **Next steps**

Bacteria composition differs by site

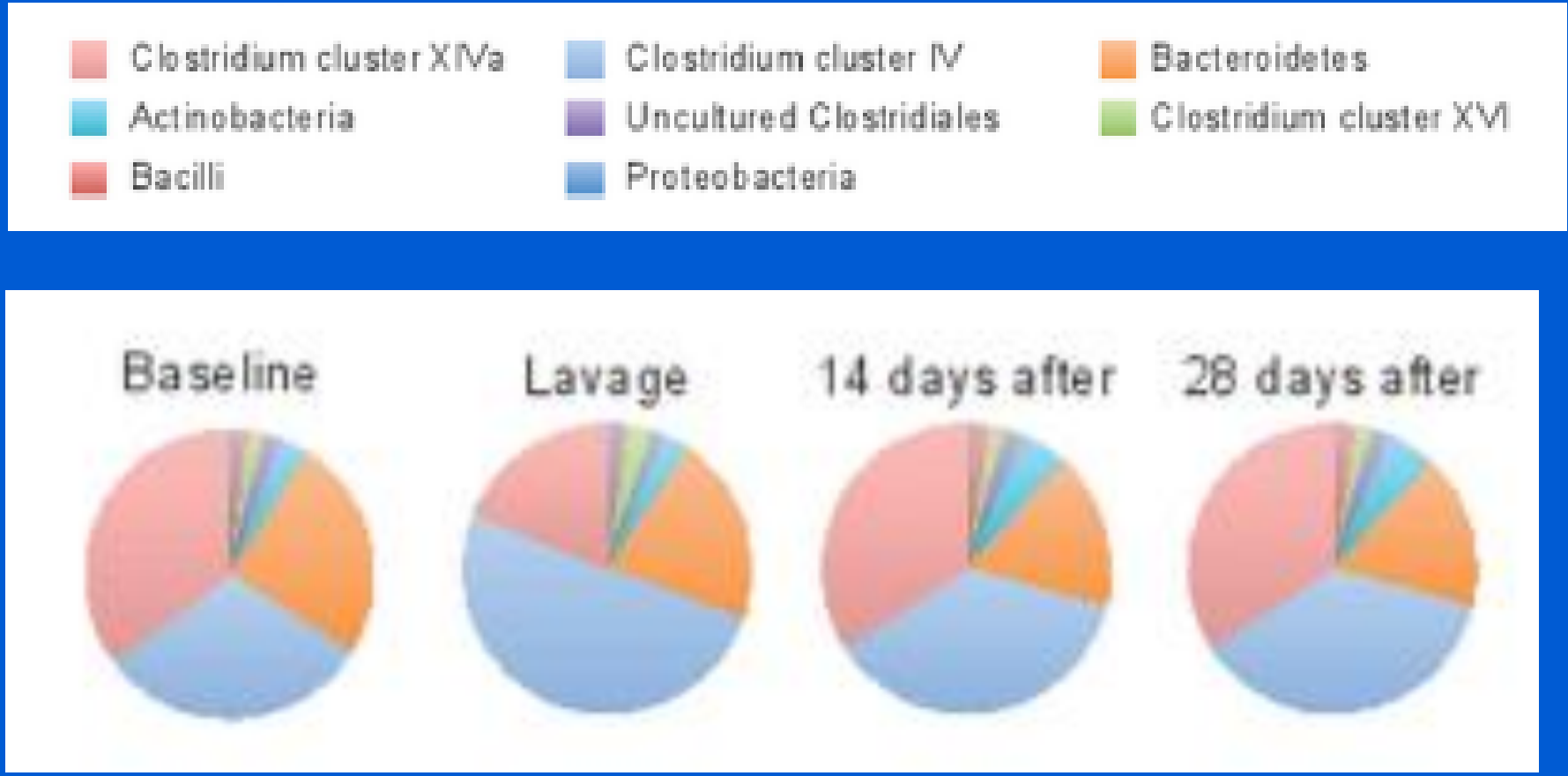


HMP Consortium, *Nature* 2012;486:207

Heterogeneity within habitats

- Gut
- Skin
- Mouth

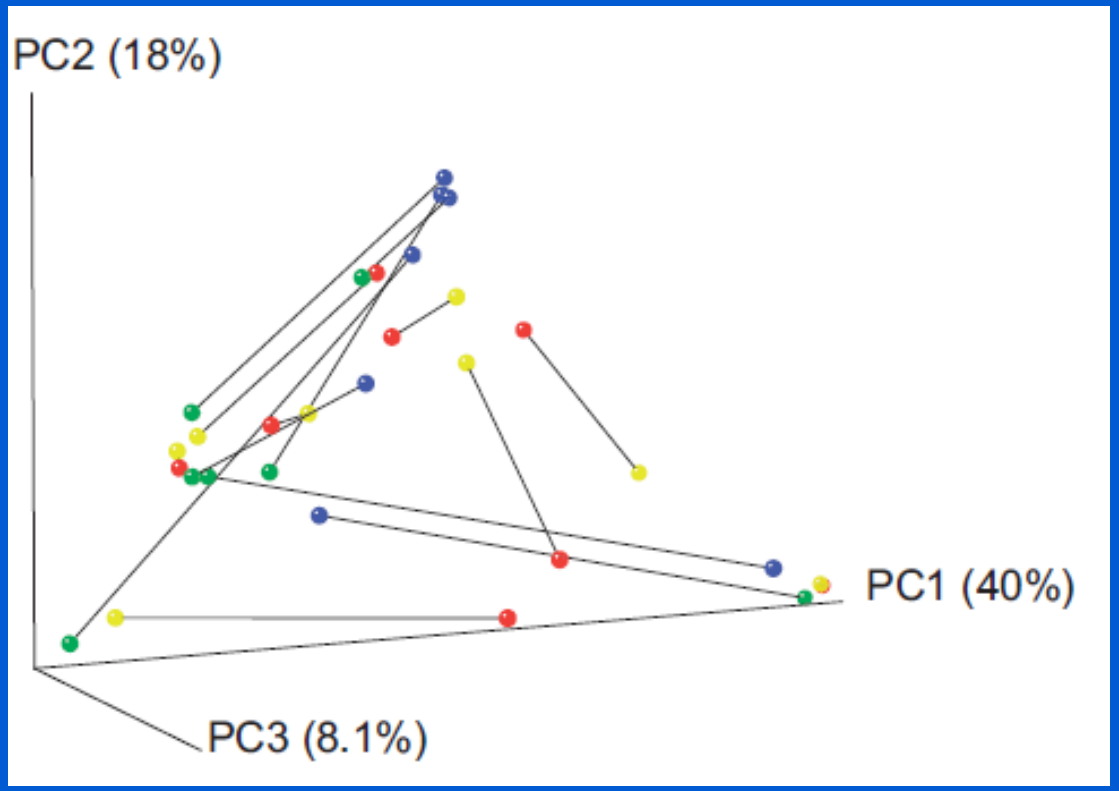
Bowel prep affects fecal microbiota



Jalanka, *Gut* 2015;64:1562

And the mucosal microbiota

Sigmoid biopsies before and after bowel preparation
Patients with IBD

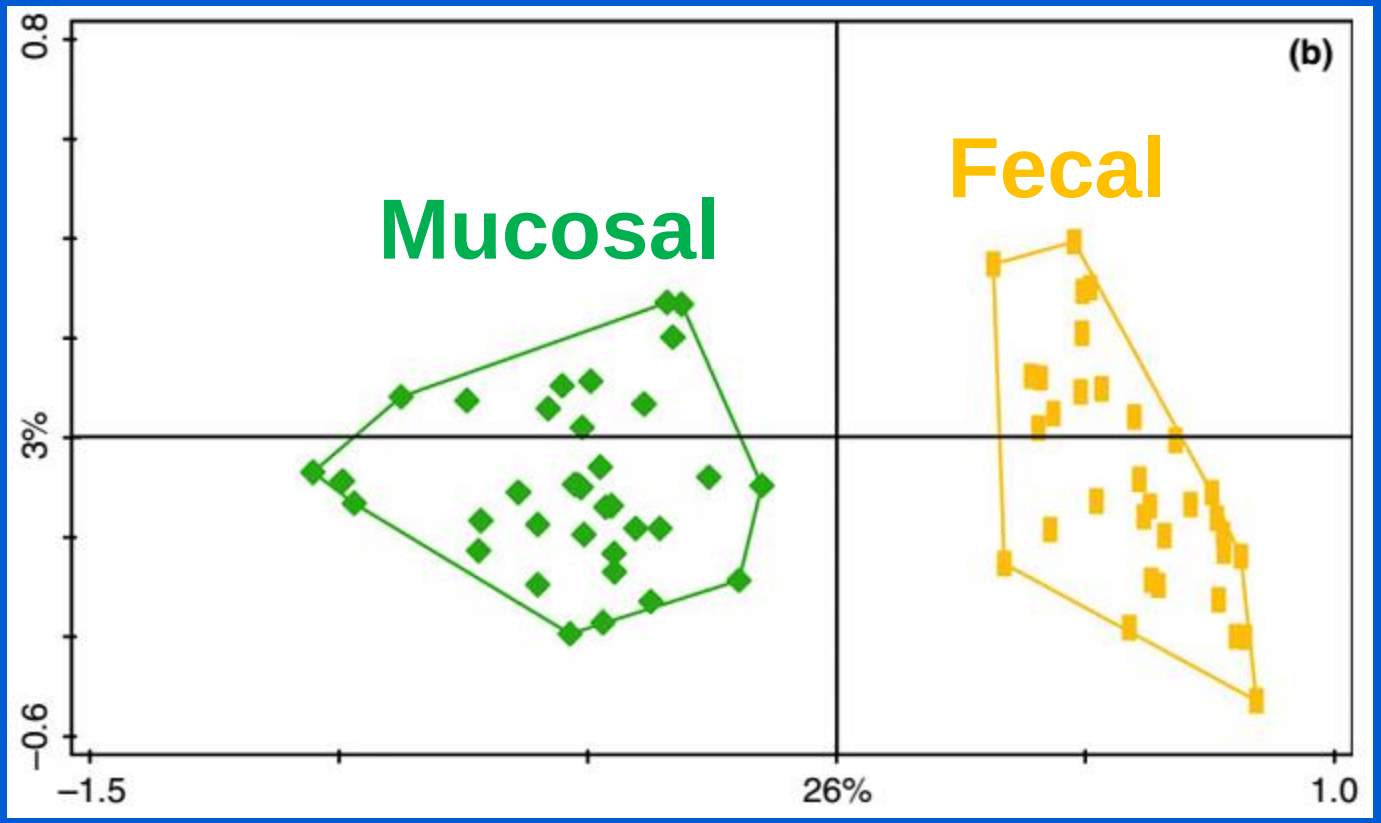


Red-yellow: Biopsy samples
Blue-green: Fecal samples

Shobar, *Clin and Transl Gastroenterol* 2016;7:e143

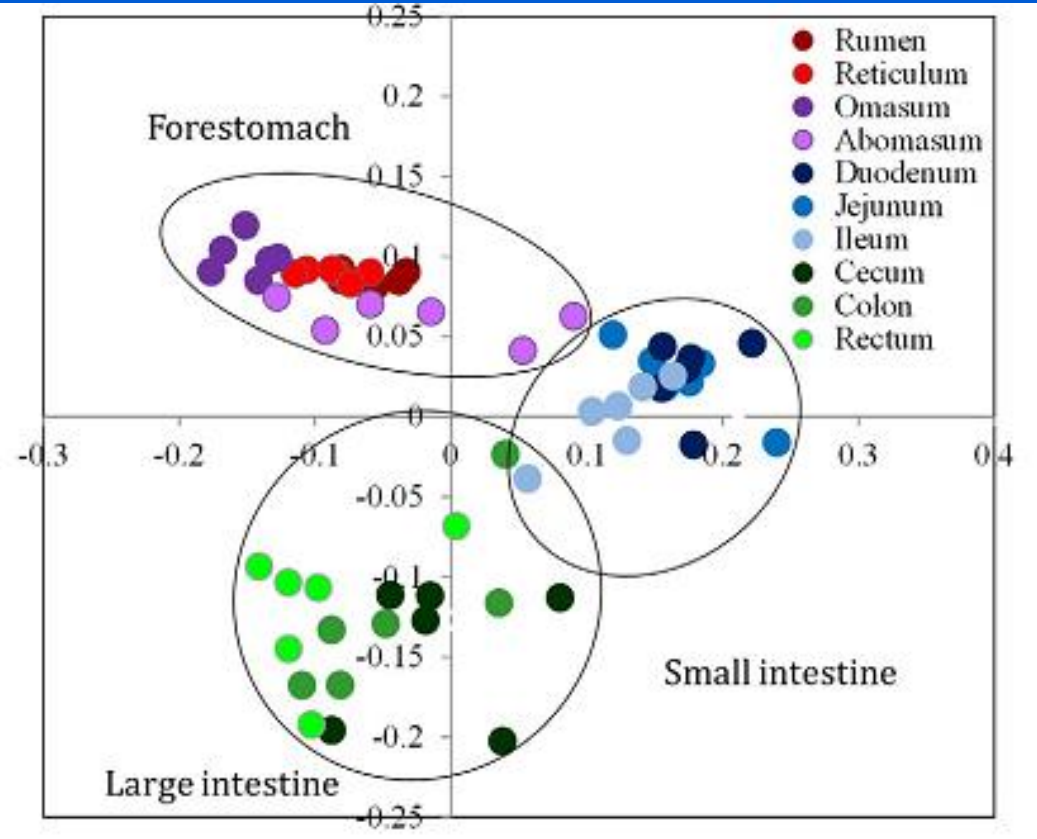
Fecal vs mucosal microbiota

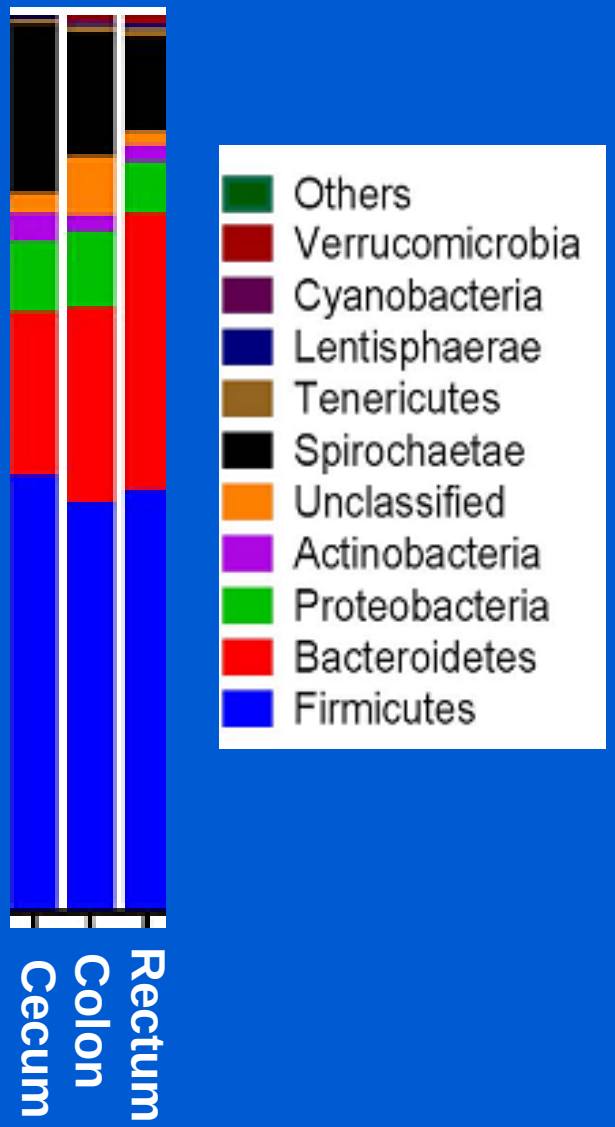
Sigmoid biopsies; no bowel prep



Rangel, *Aliment Pharmacol Ther* 2015;42:1211

Spatial heterogeneity in intestines





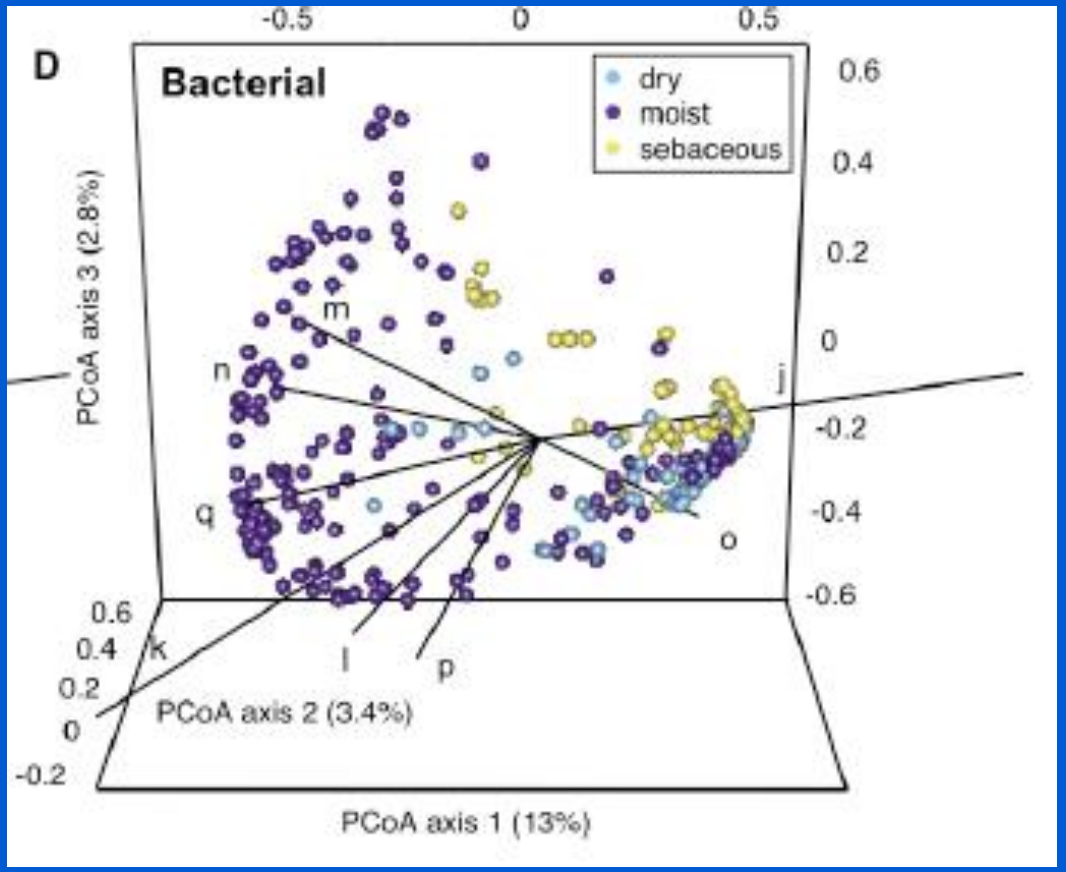
Mao, *Scientific Reports* 2015;5:16116

Skin has topographically distinct microbiota

Heel

Elbow

Back



Findley, *Nature* 2013;498:367

Mouth also has topographically distinct microbiota

Buccal mucosa
Keratinized gingiva
Hard palate

Throat
Tonsils
Tongue Dorsum
Saliva

Supragingival plaque
Subgingival plaque

Segata, *Genome Biol* 2012;13:R42

DNA preparation

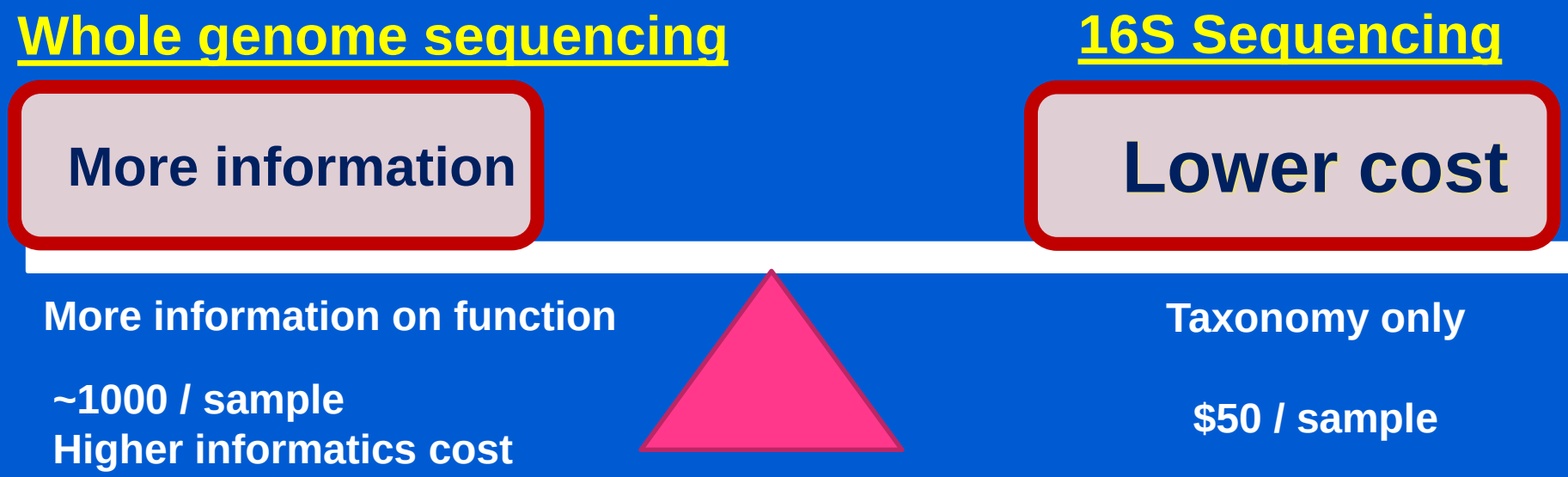
- Various kits used for DNA purification
  - MoBio preferred by HMP
  - We use Zymo for fecal collection
- Key is that you need conditions harsh enough to lyse the microbes

Outline

- Where to sample
- Sequencing
- Data analysis
- Next steps

Type of sequencing

- Amplification of 16S ribosomal DNA
- Whole genome sequencing



Sequencing prep at UAB

Starting with purified DNA

16S (Peter Eipers PhD)

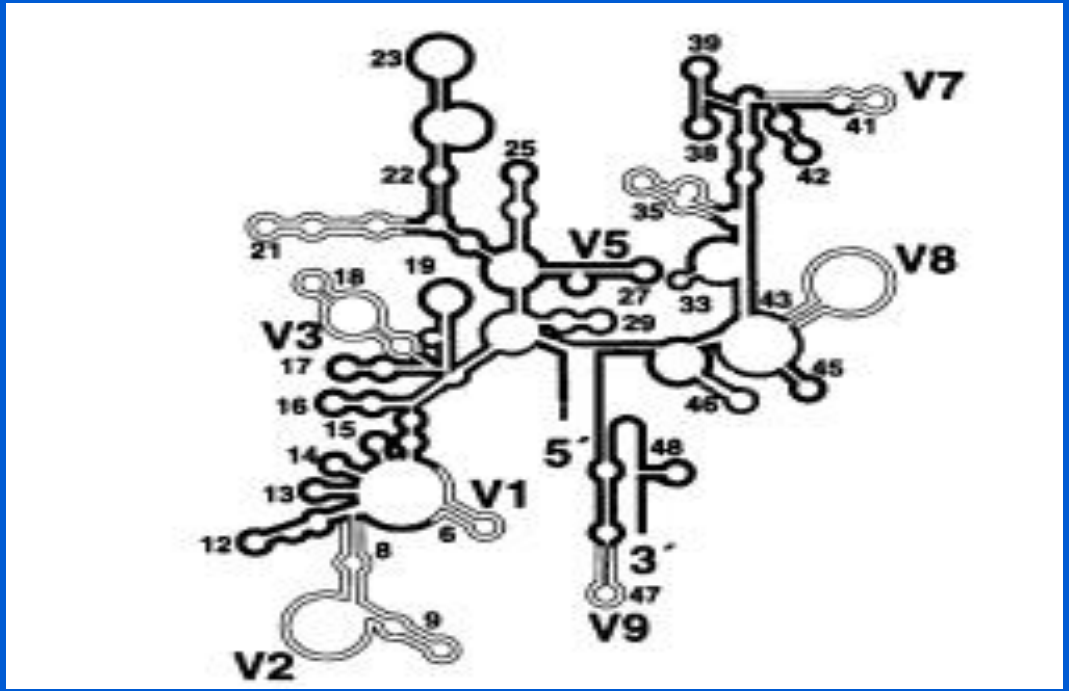
- PCR of V4 16S region
- Special primers
  - Barcode at one end
  - Adaptor at other

WGS (Mike Crowley PhD)

- Shear DNA
- Ligate adaptors to each end
  - Includes barcodes
- Short PCR
- Optional size selection

Key elements of 16S rDNA

- 9 conserved and 9 variable regions
  - Interspersed
- Primers bind to conserved regions
- Amplify variable sequences



Tortoli, *Clin Microbiol Rev* 2003;16:319

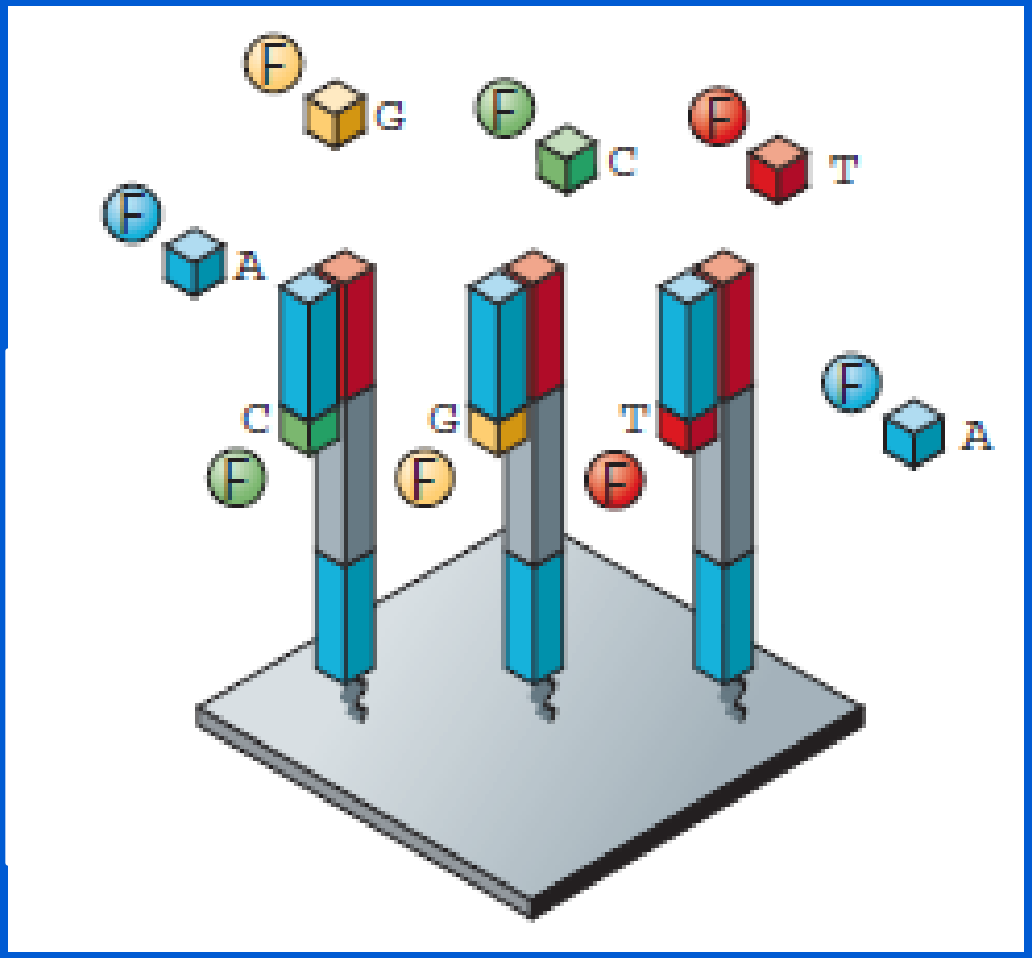
Foundation of 16S analysis

Position	Consensus composition	Purple bacteria				Gram-positive bacteria		Cyano-bacteria	Green sulfur bacteria	Spirochetes		Bacteroides		Planc-tomyces	Deino-coccus	Green non-sulfur bacteria
		α	β	γ	δ	Lo	High			Spiro-chetes	Lepto-spiras	Bacter-oides	Flavo-bacteria[a]			
47	C	●[b]	●	●	●	●	●	●	●	U[c]	U	●	●	G	●	●
48	Y	●	●	●	●	●	●	●	●	●	●	●	●	A	●	●
50	A	●	U	●	●	●	●	●	●	U	U	●	●	U	●	●
52	Y	●	●	●	●	●	●	●	●	A	A	●	●	G	G	●
53	A	●	●	●	●	●	●	●	●	G	●	●	●	G	●	G
353	A	●	●	●	●	●	●	●	●	●	●	●	●	U	●	●
570	G	●	●	●	●	●	●	●	●	●	●	U	U	U	●	●
698	G	U	●	●	Y	●	●	●	●	●	●	●	●	●	●	●
812	G	●	C	●c[d]	●	●	●	●	●	●	C	●	●c	●	C	●
906	G	Ag	Ag	Ag	Ag	●a	A	●	●	●	●	●	●	●	●	A
933	G	●	●	●	●	●	●	●	●a	●	●	●	●	A	●	●
955	U	●	Au	●	●	●	AC	●	●	●	●	●	●	C	●	●
976	G	●	A	●	●	●	●	●	●	●a	A	●	●	●	●	●
983:1	—[e]	—	—	—	—	—	—	—	—	—	—	—	—	U	—	—
995	C	●	●	●	●	●	●	●	(A)[f]	●	●	A	A	●	●	●

Carl Woese, *Microbiol Rev* 1987;51:221

Cyclic reversible termination

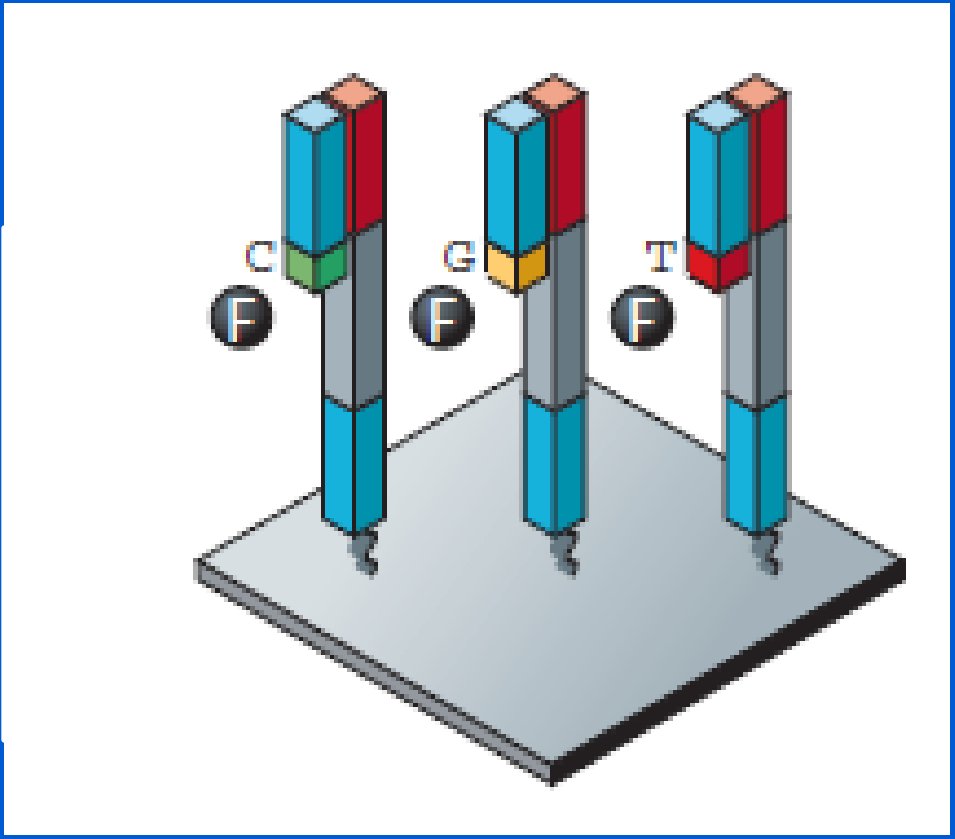
Metzker, *Nature Rev Genet* 2010;11:31



Incorporate all four nucleotides, each with different dye

Cyclic reversible termination

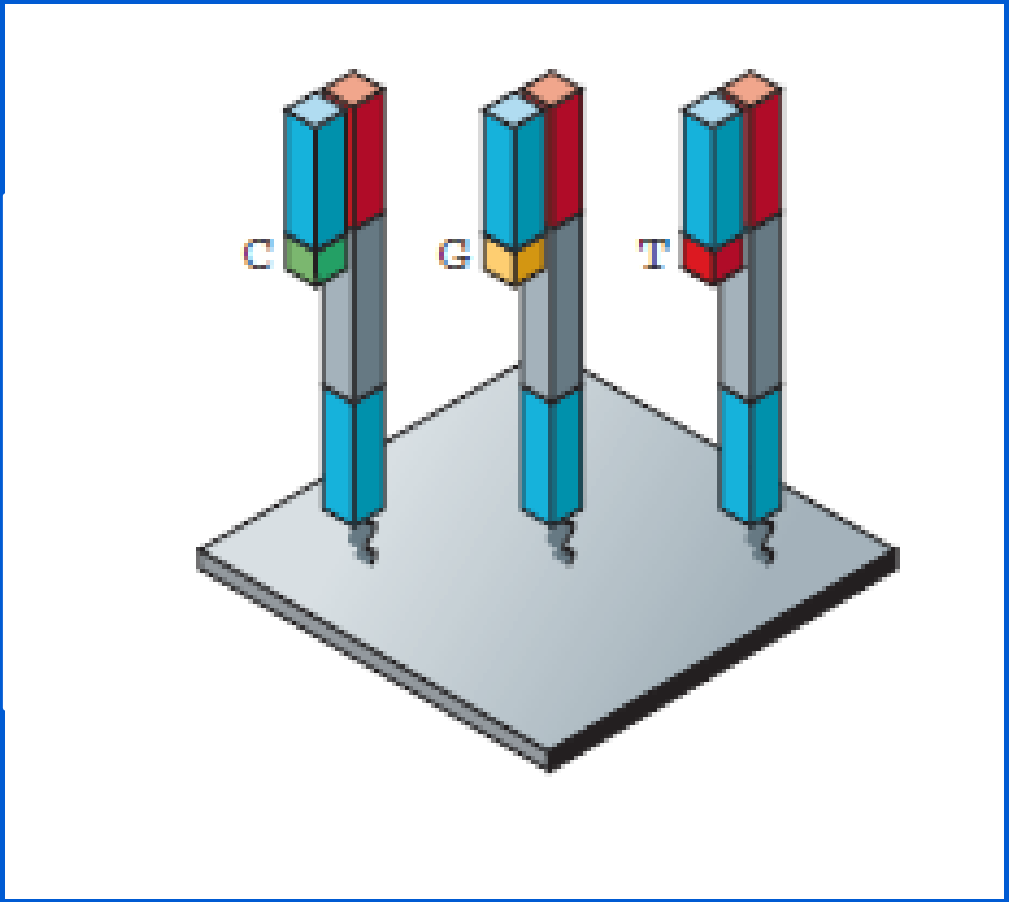
Metzker, *Nature Rev Genet* 2010;11:31



Wash out unused nucleotides; image

Cyclic reversible termination

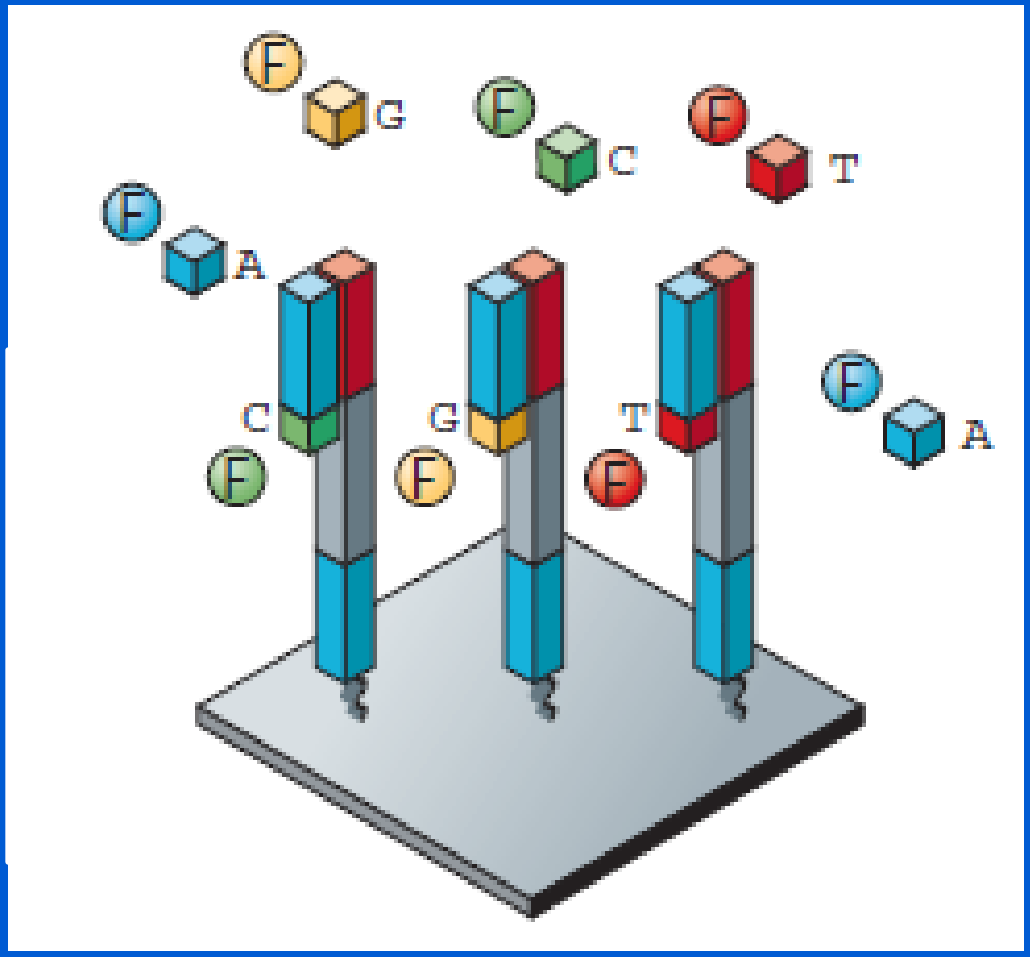
Metzker, *Nature Rev Genet* 2010;11:31



Cleave dye and terminating groups

Cyclic reversible termination

Metzker, *Nature Rev Genet* 2010;11:31



Repeat

Barcodes to sort out samples

Sample 1: AGGTTCCA
Sample 2: GGCAATTT
Sample 3: TTGGAAAC

Outline

- Where to sample
- Sequencing
- Data analysis
- Next steps

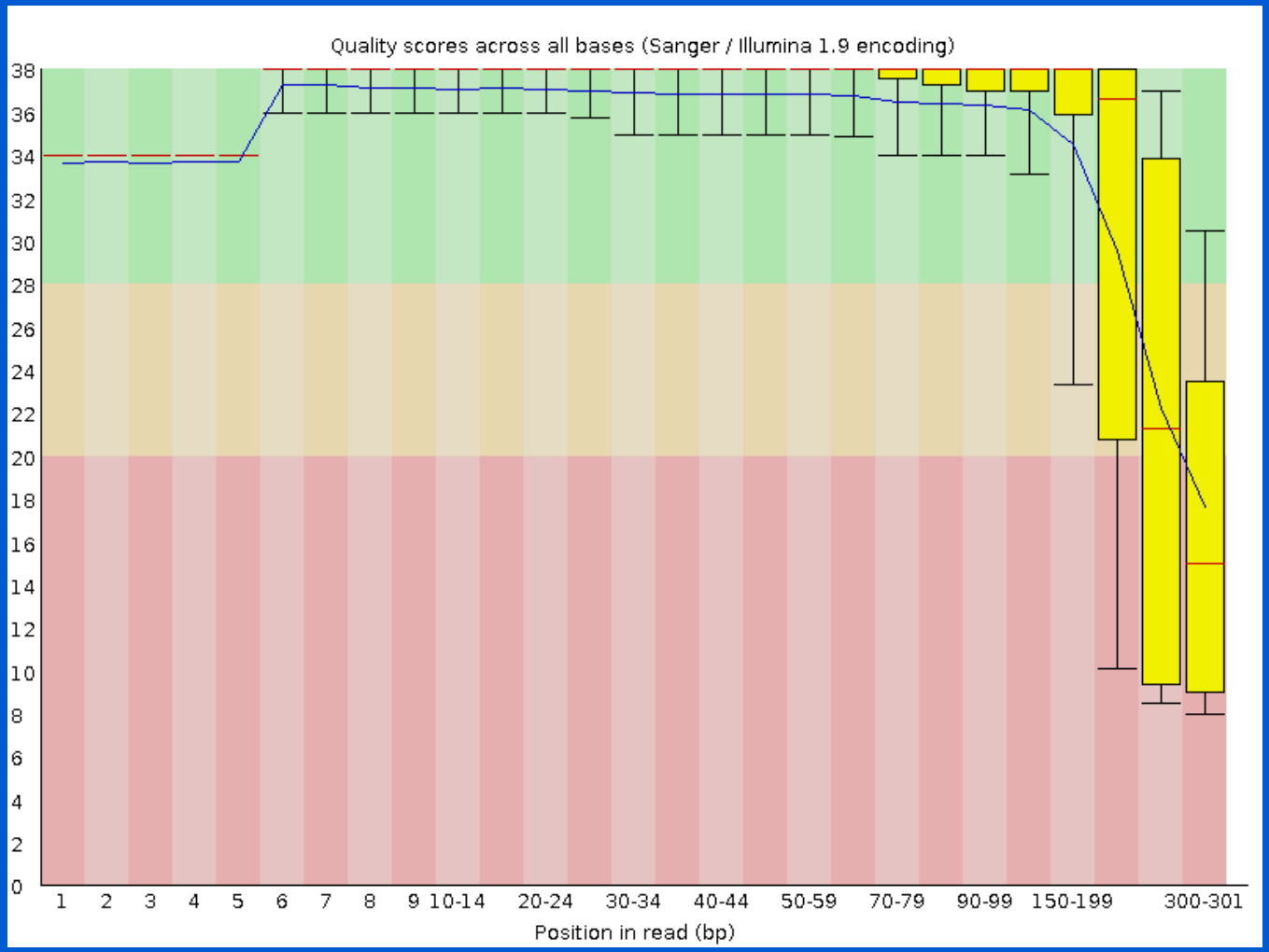
Output: fastq files

Sample Fastq output (two DNA strands)

Header: @M02079:147:000000000-AK0J5:1:1101:15736:1676 1:N:0:49
Sequence: TACAGAGGTCTCAAGCGTTGTTCGGAATCACTGGGCGTAA
Additional line: +
Quality: >//>>EEGGFFE/////</>-<0>DBF1<F<1.<<-<GD

Header: @M02079:147:000000000-AK0J5:1:1101:15989:1722 1:N:0:49
Sequence: TACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAG
Additional line: +
Quality: B3EHFGGHF3FB43/E?EFGGFFGH3/B4?//B/FG?122FB

Assess quality of reads



Position in read (BP)

Quality filtering options

- **Trim the low-quality tails**
  - **Option: remove sequence if more than a set percentage of bases are trimmed**
- **Remove sequences with:**
  - **Ambiguous bases**
  - **Quality scores below a threshold**
  - **More than a certain number of predicted errors**

The bane of amplicon analysis is PCR errors

- PCR errors, especially if they occur early in the cycle, can look like unique species
- Additionally, sequencing errors may have occurred, especially if the quality is low
- A common approach to addressing this issue is clustering of similar sequences into operational taxonomic units (OTUs)

QIIME

- Open-source bioinformatics pipeline
- Designed for 16S sequence analysis
- Every step from fastq processing through data analysis

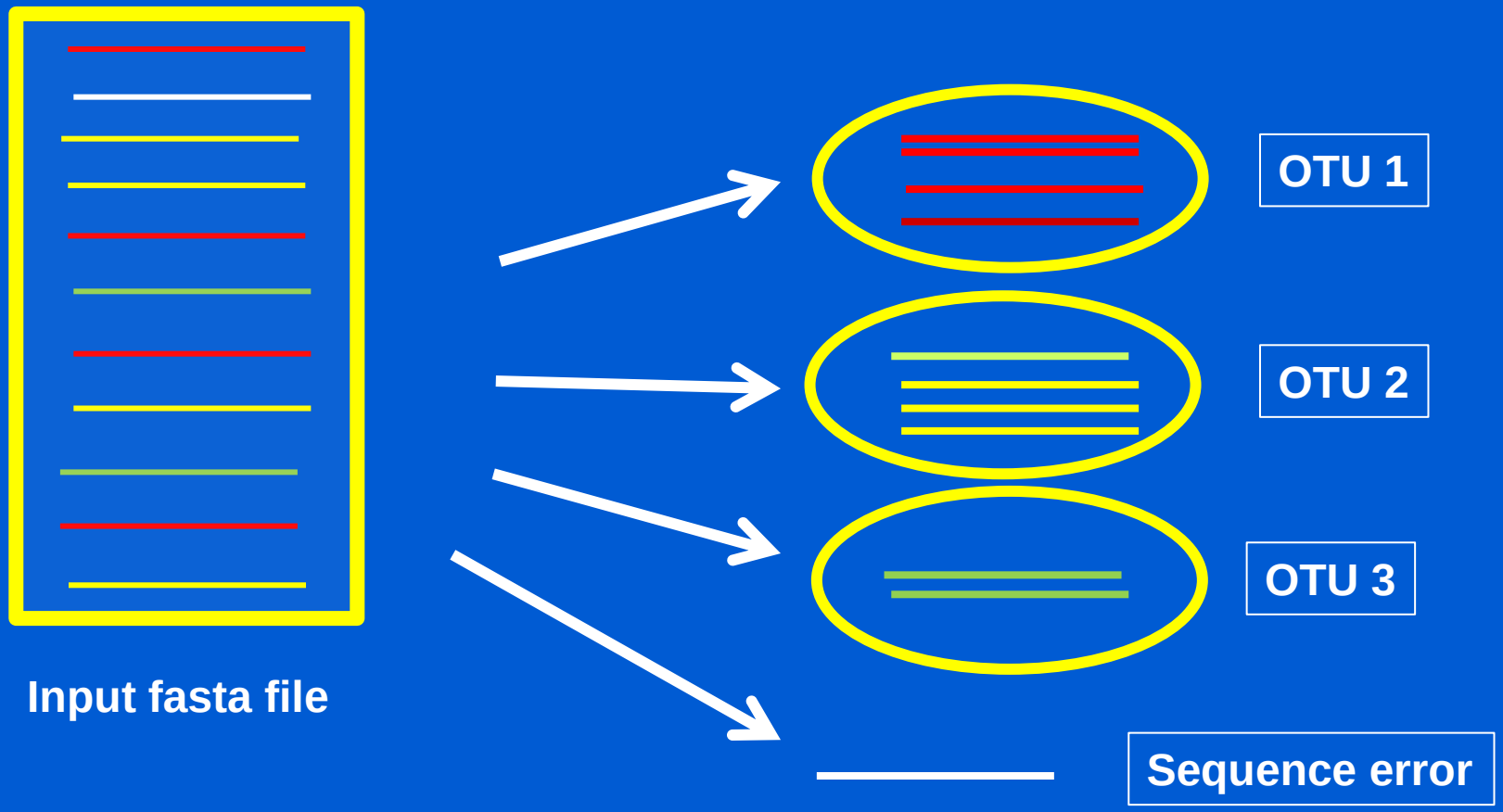
www.QIIME.org

Operational taxonomic unit picking

- Similar (typically 97%) sequences are clustered together
- Creates an "OTU table"
  - List of all OTUs
  - Frequency of each OTU in each sample
  - Taxonomic assignment of each OTU

OTUId	Sample1	Sample2	Sample3	Taxonomy
OTU_1	30	70	35	k__Bacteria;p__Firmicutes…
OTU_2	40	10	40	k__Bacteria;p__Bacteroidetes…
OTU_3	30	20	25	K__Bacteria;p__Firmicutes…

Basic idea of OTU picking



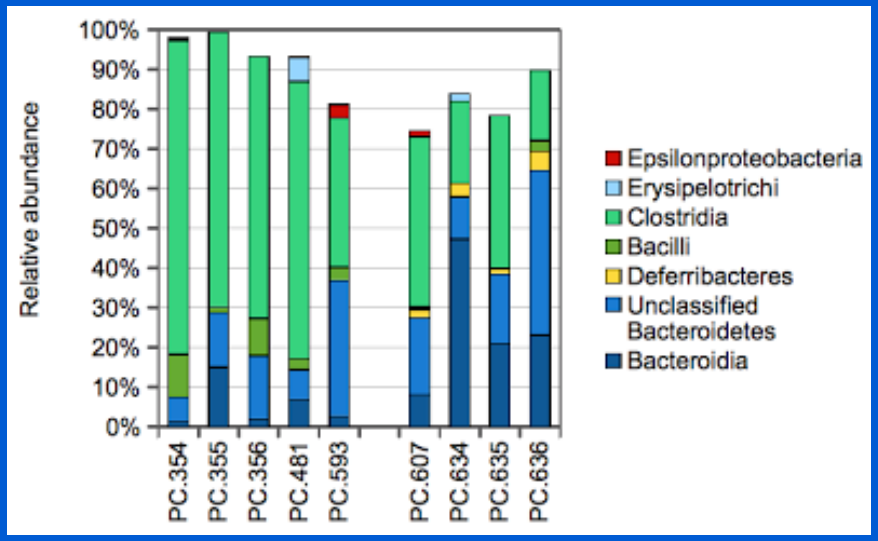
Limitations to OTU picking

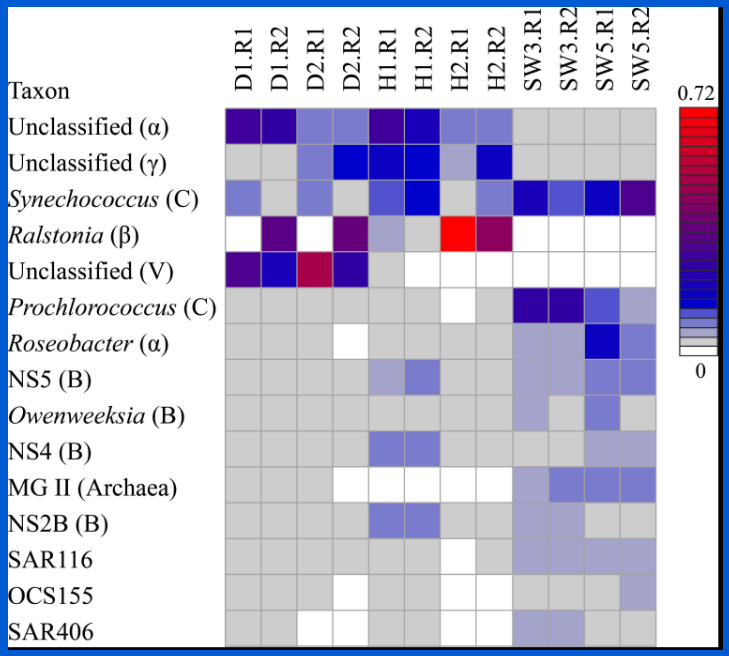
- OTUs are clustered based upon similarity
- Strain- and even species-level distinctions are often lost
- PCR amplification errors and low quality reads with high error rates can be mistaken as unique OTUs

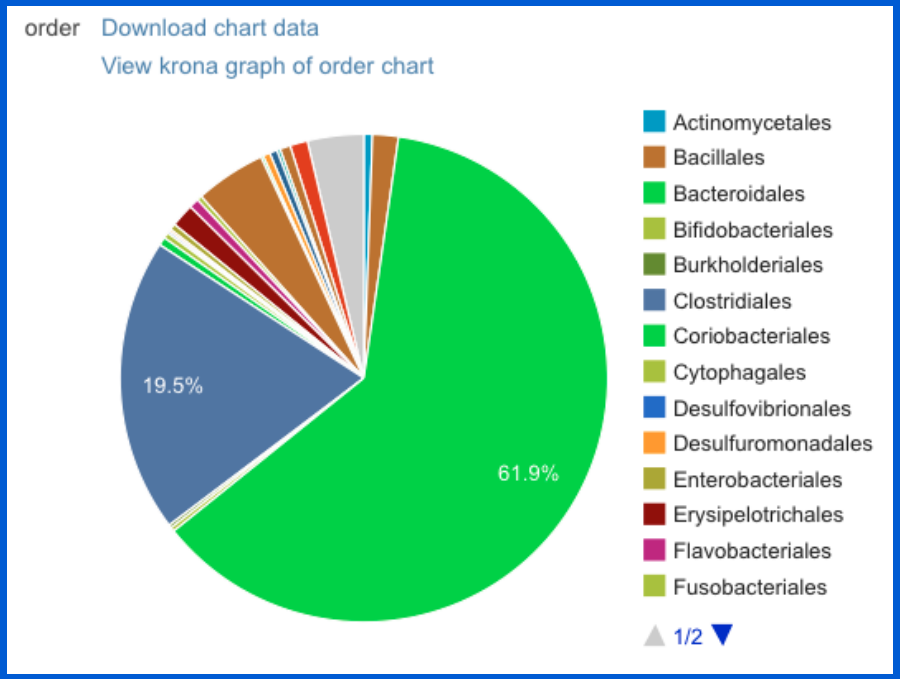
Downstream analyses with OTU tables

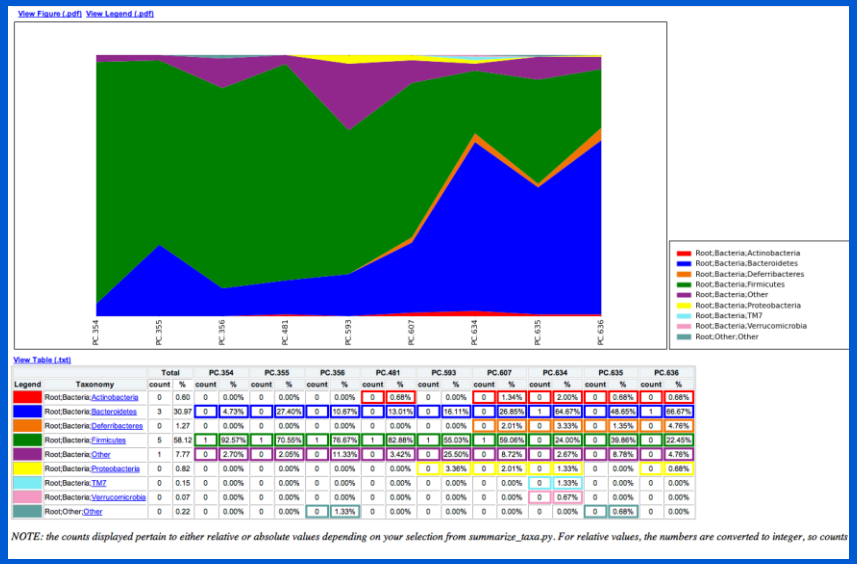
- Display taxonomy
- Alpha diversity (within group)
- Beta diversity (between groups)

Displaying taxonomy

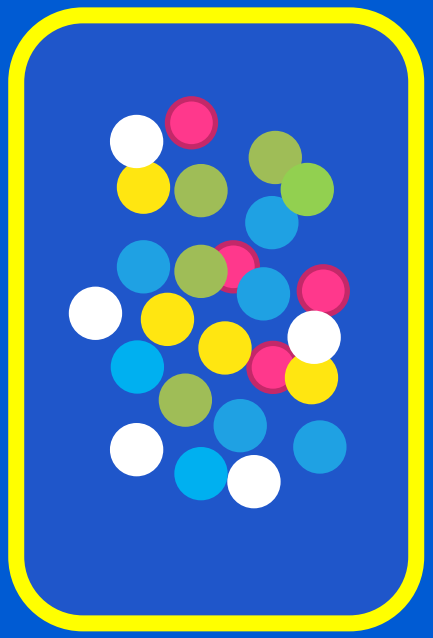




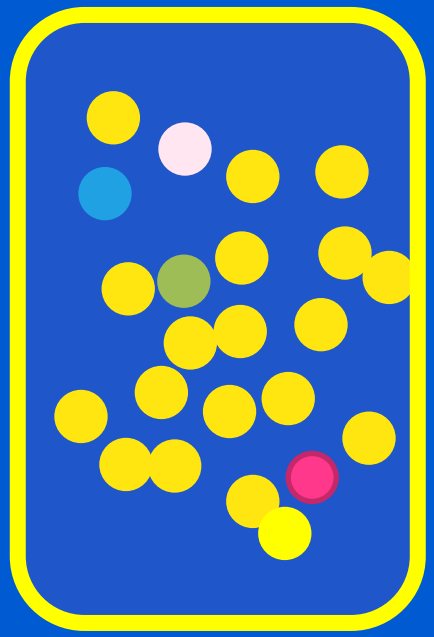




Alpha diversity



Rich and even



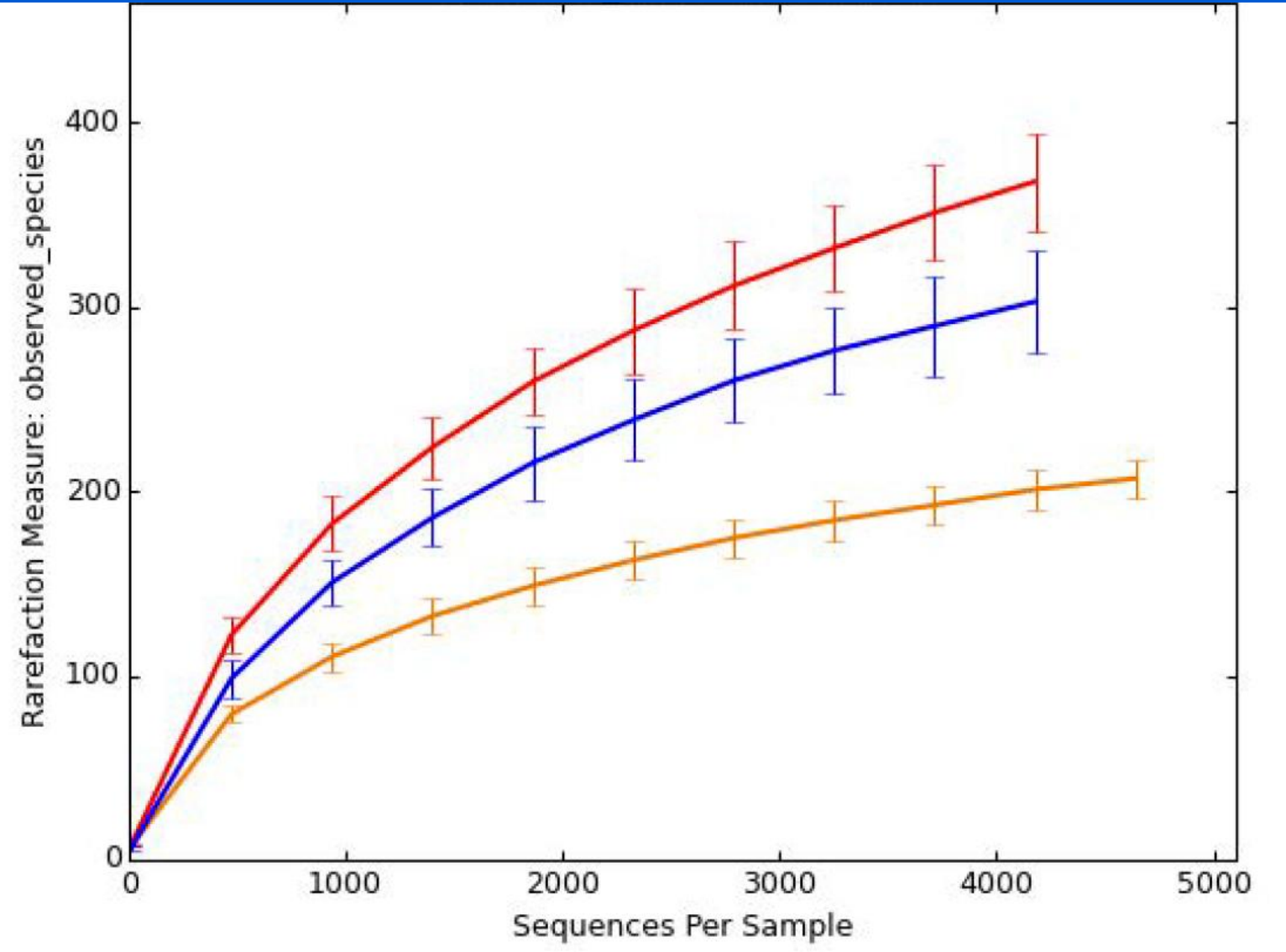
Rich, not even

Not rich or even

Cautionary note about measuring alpha diversity

- Must take into account sequencing depth
- Typically, at UAB, depth is 50K – 150K sequences per sample for 16S
- To a point, diversity increases with higher depth, as you pick up more rare species

Illustration of rarefaction curves



Beta diversity

- This speaks to the diversity between two different groups
- Typically depicted as PCOA plots
- Consider sequencing depth

Sample distance matrix

	C1	C2	C3	C4	T1	T2	T3
C1	0	0.35	0.31	0.39	0.88	0.79	0.91
C2	0.35	0	0.42	0.22	0.92	0.90	0.74
C3	0.31	0.42	0	0.35	0.74	0.79	0.91
C4	0.39	0.22	0.35	0	0.82	0.84	0.92
T1	0.88	0.92	0.74	0.82	0	0.29	0.21
T2	0.79	0.90	0.79	0.84	0.29	0	0.32
T3	0.91	0.74	0.91	0.92	0.21	0.32	0

Sample distance matrix

	C1	C2	C3	C4	T1	T2	T3
C1	0	0.35	0.31	0.39	0.88	0.79	0.91
C2		0	0.42	0.22	0.92	0.90	0.74
C3			0	0.35	0.74	0.79	0.91
C4				0	0.82	0.84	0.92
T1					0	0.29	0.21
T2						0	0.32
T3							0

Statistical tests compare distance between vs within groups

e.g. Permanova

Microbiota in juvenile spondyloarthritis

Spondyloarthritis

- Type of pediatric and adult arthritis
- Distinctive demographic & clinical features
- High prevalence of gut inflammation
  - Frank IBD: 5 – 10% (IBD-associated arthritis)
  - Subclinical: 67%
- In children, goes by the name enthesitis-related arthritis (ERA)

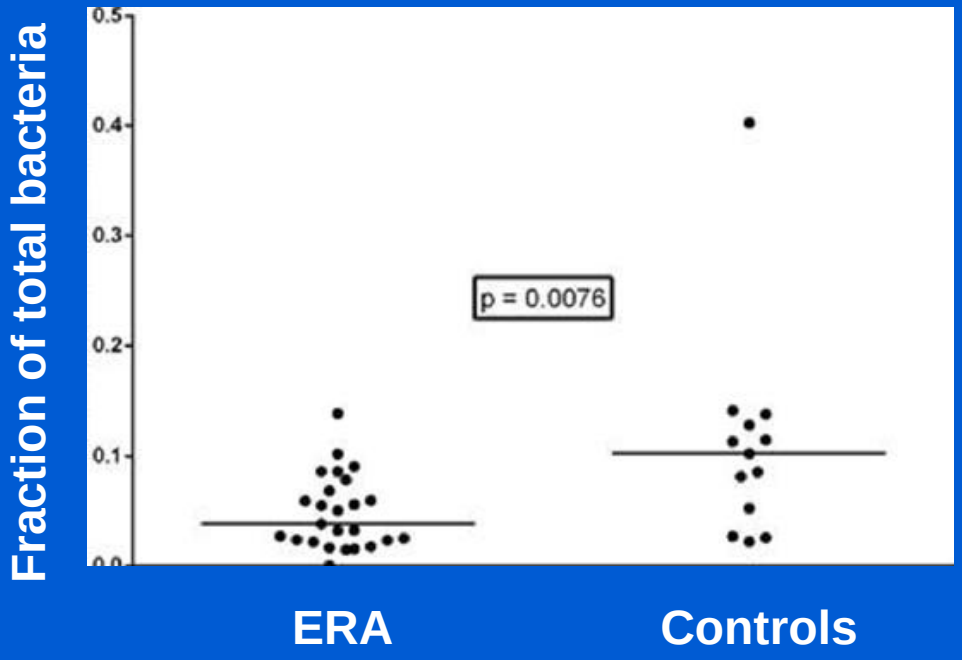
Initial foray into microbiota analysis

- Obtained fecal specimens from children with ERA, along with healthy controls
- Patients included those who were newly diagnosed as well as those with long-standing disease
- Performed sequencing of 16S rDNA
- Analyzed with QIIME platform

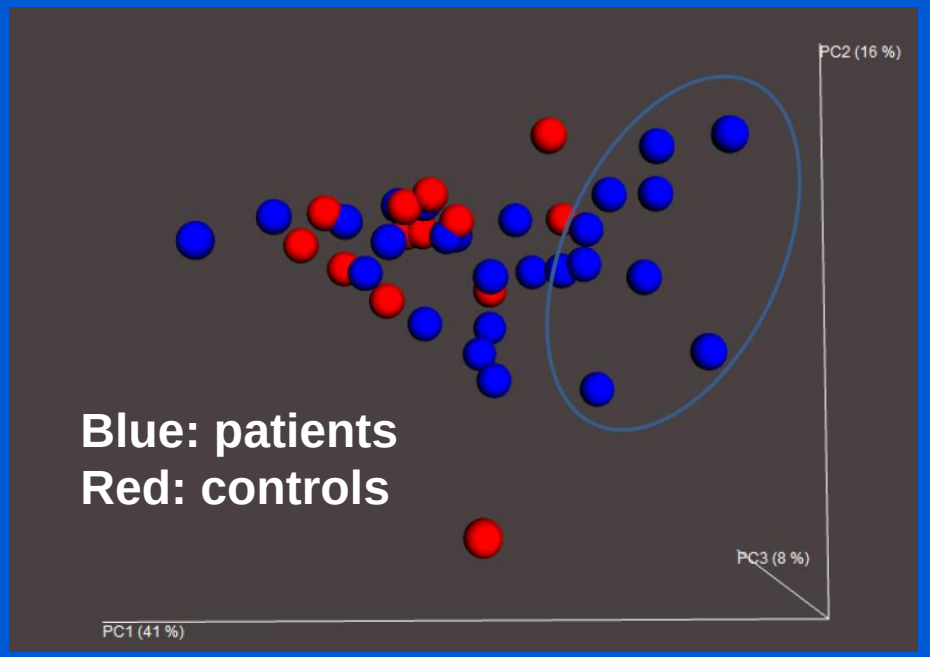
Microbiota in ERA

- Decreased *Faecalibacterium prausnitzii* in children with ERA
- Small patient cluster identified by PCoA

Fecal abundance of *F. prausnitzii*



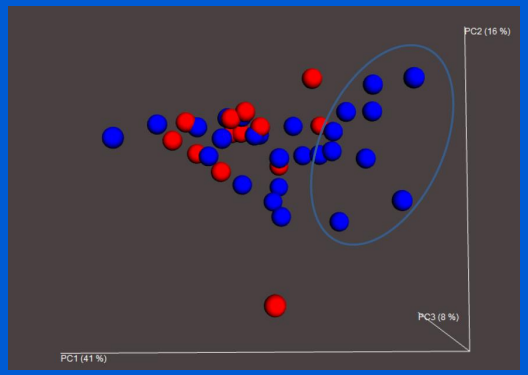
Principal coordinates analysis



Stoll, *Arth Res Ther* 2014;16:486

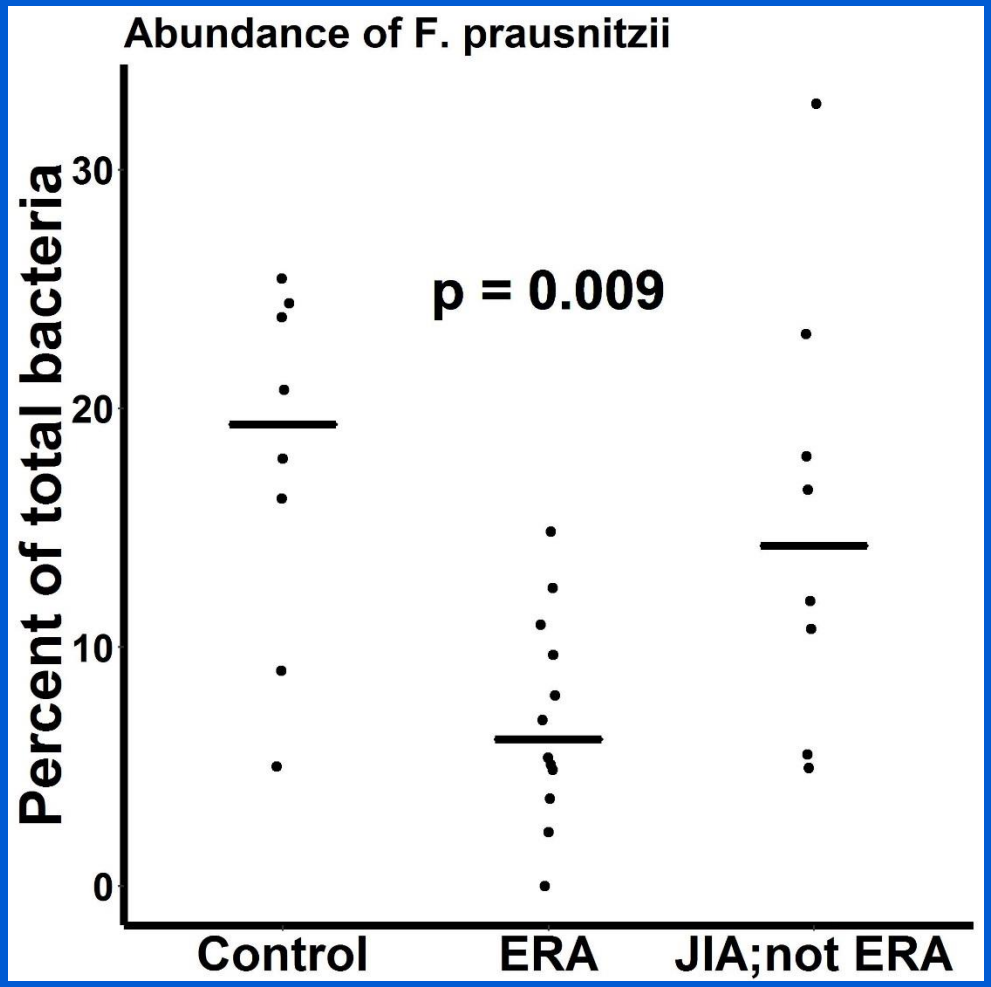
Clusters are defined by *Bacteroides* and *Akkermansia*

- **The two clusters had similar abundance of *F. Prausnitzii***
- **The subjects who clustered with the controls tended to have high abundance of *Akkermansia* (> 1% in 7/17 vs 0/8)**
- **The subjects forming their own cluster had high *Bacteroides* abundance (41% vs 13%)**



Stoll, *Arth Res Ther* 2014;16:486

Altered *F. prausnitzii* is largely limited to SpA subtype of pediatric arthritis



Stoll, *Clin Immunol* 2015;159:134

F. prausnitzii is an anti-inflammatory organism

- **Decreased fecal abundance in IBD[1]**
- **Increased IL-10 production by PBMCs[2,3]**
- **Reduces inflammation in colitis model[2]**
- **Major butyrate producer[4]**
  - **Beneficial effects on enterocytes[5]**
  - **Increases colonic regulatory T cells[6]**

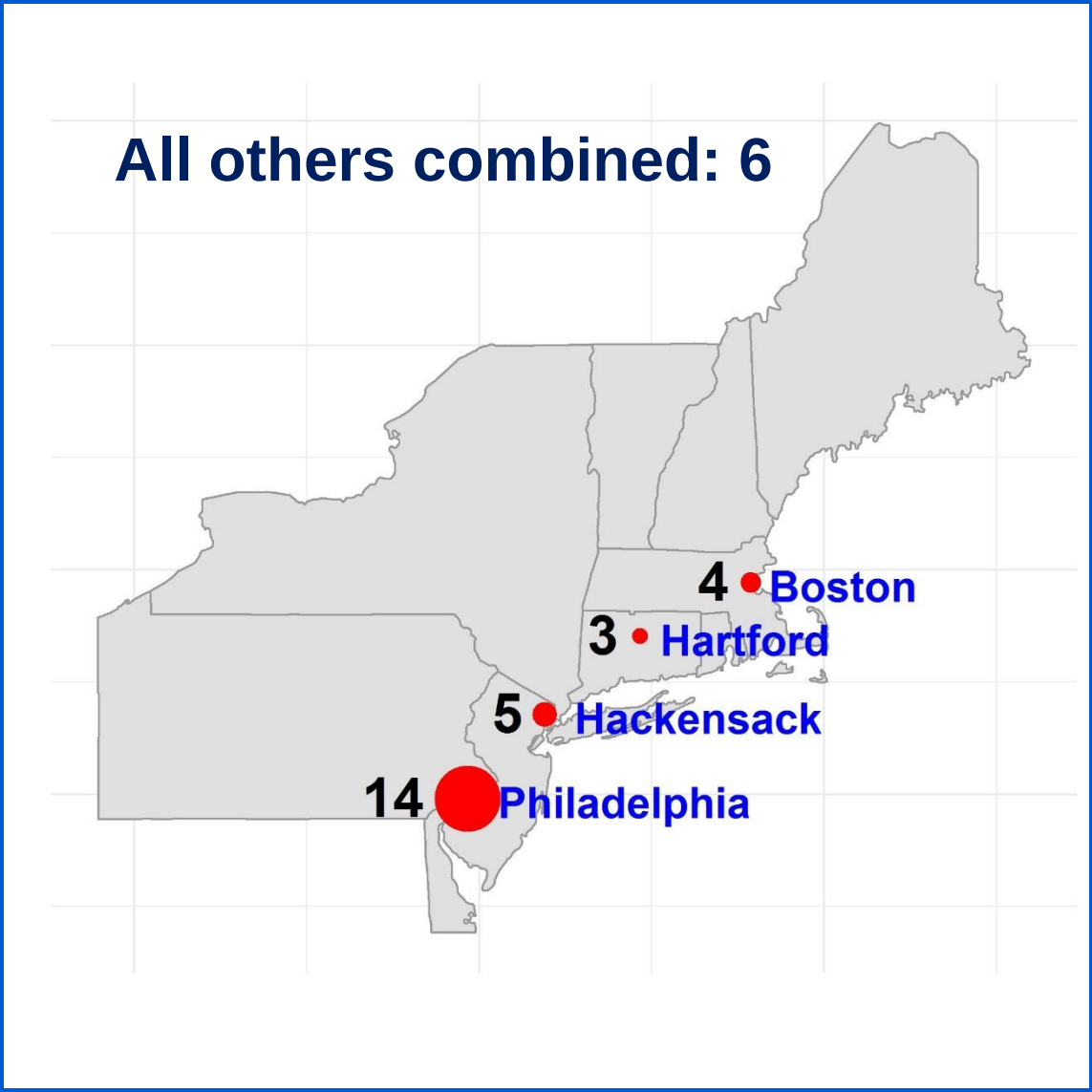
[1]Cao,*Gastroenterol Res Practice* 2014:872725 (review)
[2]Sokol, *PNAS* 2008;105:16731
[3]Rossi, *Sci Rep* 2016;6:18507
[4]Hold, *Appl Environ Microbiol* 2003;69:4320
[5]Hamer, *Aliment Pharmacol Ther 2008;27:104*
[6]Smith, *Science* 2013341:569

Focus on treatment-naïve subjects

- Recruited pediatric spondyloarthritis patients from centers around the country
- Attempted to recruit healthy controls from each site
- Analyzed with QIIME (initially)

Collaborating centers

Collaborators are not all created equal



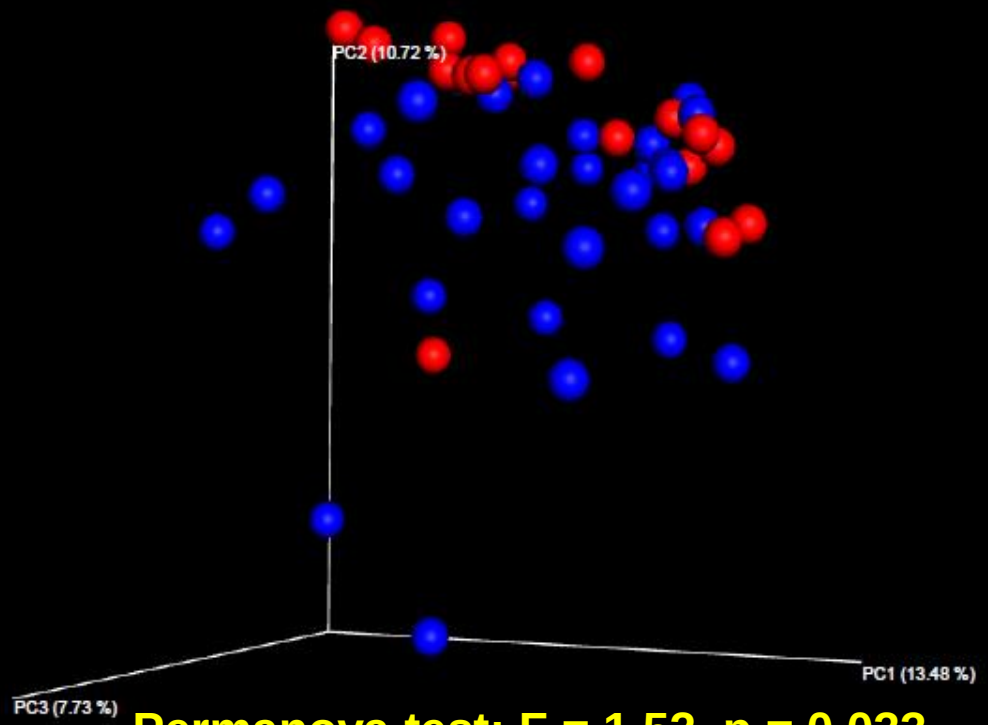
Subjects

Characteristic	ERA	Controls
n	30	19
Age (years; mean ± SD)	13.5 ± 3.0	13.6 ± 2.7
BMI (kg / m^2; mean ± SD)	20.7 ± 4.1	21.5 ± 6.1
Male	19, 63%	13, 68%
Caucasian	23, 77%	17, 89%

Clustering by diagnosis

Blue: patients
Red: controls

Bray Curtis

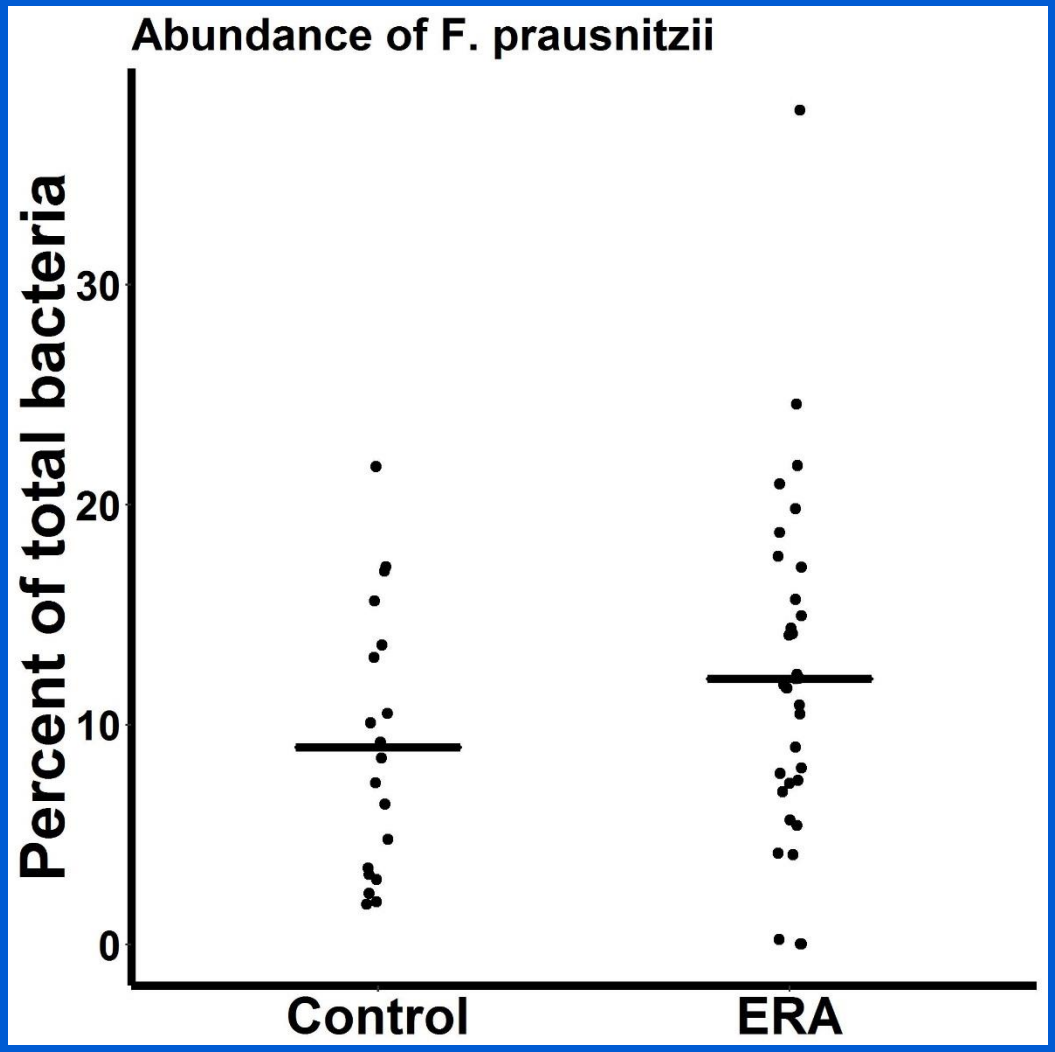


Permanova test: F = 1.52, p = 0.033

Taxonomic assessment

- **Pairwise comparisons revealed minimal differences between the groups**
  - **Especially after adjustment for multiple comparisons**
  - **At the phylum level, decreased Actinobacteria in ERA (3.3 vs 9.0%, $p < 0.002$)**
- **Focused on organism of interest based upon previous work**
  - ***Faecalibacterium prausnitzii***

Not what I expected



Limitations to OTU picking

- OTUs are clustered based upon similarity
- Strain- and even species-level distinctions are often lost
- PCR amplification errors and low quality reads with high error rates can be mistaken as unique OTUs

Alternative to OTU picking

Published in final edited form as:
Nat Methods. 2016 July ; 13(7): 581–583. doi:10.1038/nmeth.3869.

DADA2: High resolution sample inference from Illumina amplicon data

Benjamin J Callahan[1,*], **Paul J McMurdie**[2], **Michael J Rosen**[3], **Andrew W Han**[2], **Amy Jo A Johnson**[2], and **Susan P Holmes**[1]

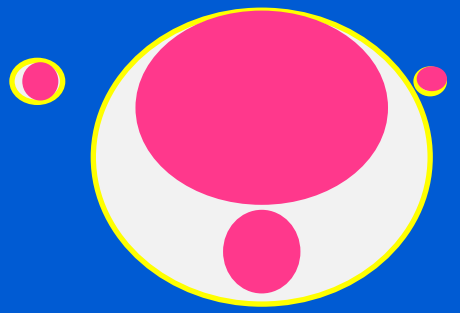
[1]Department of Statistics, Stanford University, Stanford, CA, USA

[2]Second Genome, South San Francisco, CA, USA

[3]Department of Applied Physics, Stanford University, Stanford, CA, USA

- **Use error modeling to distinguish sequence errors from sequence variants**
  - **Real OTUs versus fake OTUs**

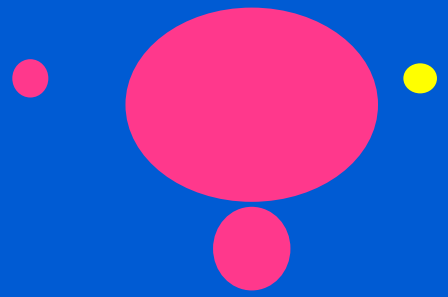
DADA2 offers a replacement to traditional OTU picking methods



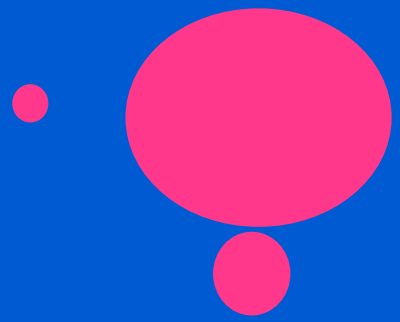
DADA2 – basic concept

- Start with the most abundant sequence (centroid)
- For each other sequence, test the null hypothesis that it is the same as the centroid
- Two factors are used to compare sequences with the centroids
  - Distance
    - Number of nucleotide differences
    - Quality of the sequences
    - Specific nucleotide transitions (e.g. T → A, T → C, etc)
  - Abundance
- If the lowest (smallest) p-value is below a cutoff, then the null hypothesis is rejected, and the sequence becomes a centroid
- Otherwise, the sequence is combined with the nearest centroid

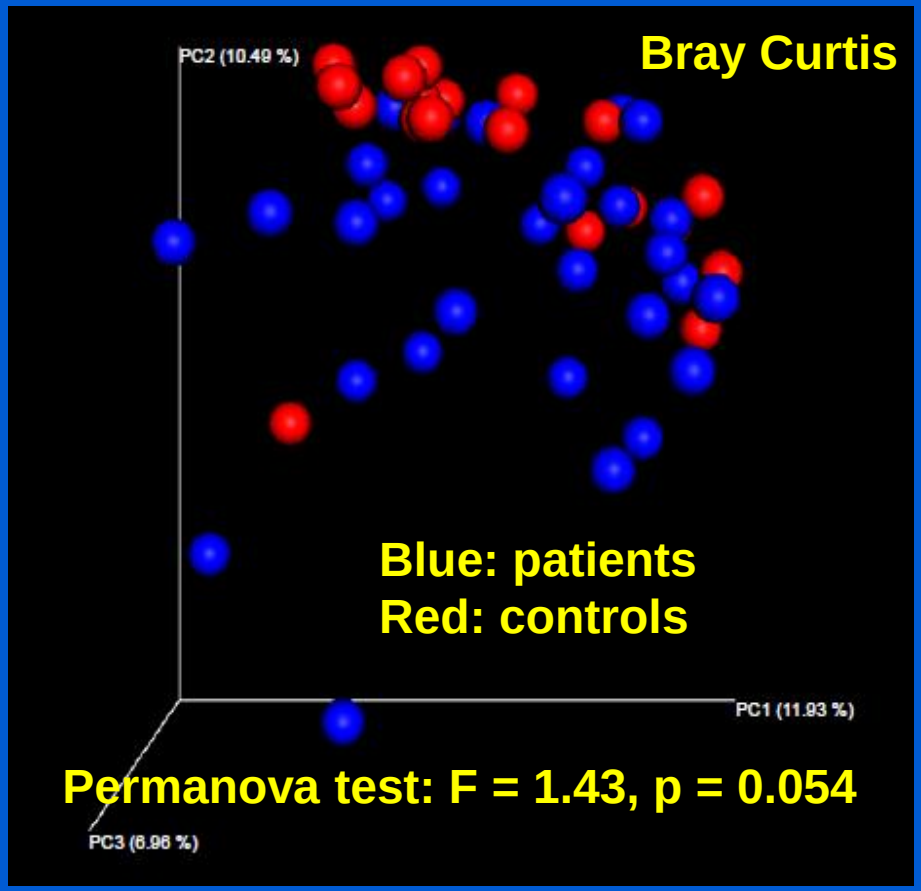
DADA2 offers a replacement to traditional OTU picking methods

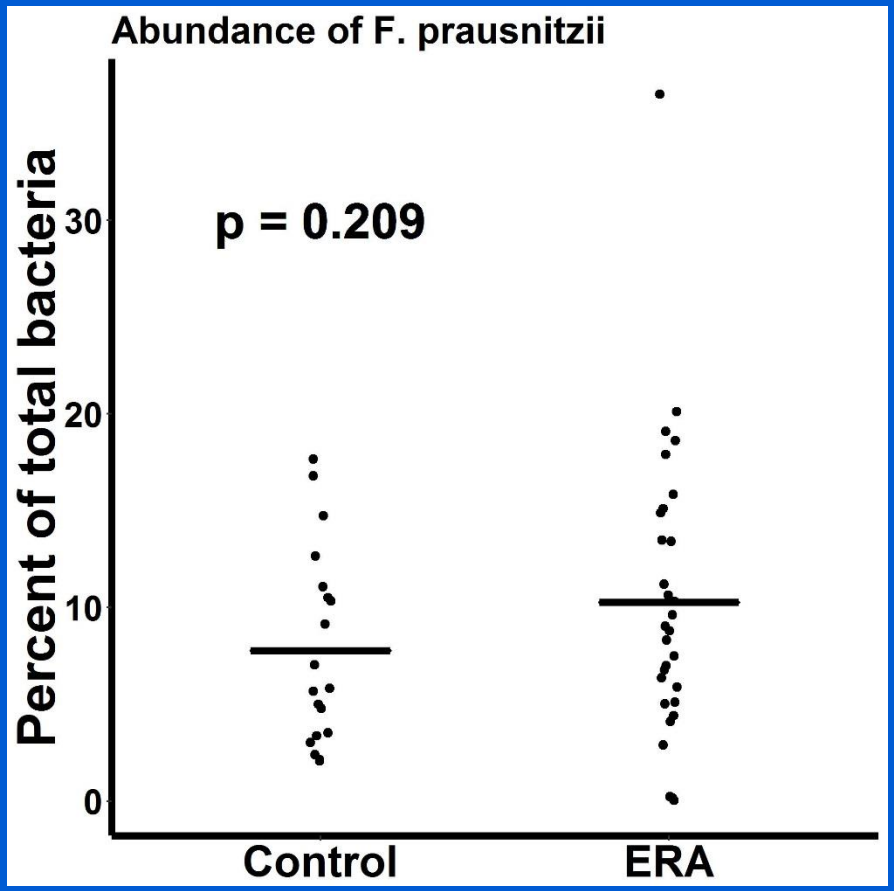


DADA2 offers a replacement to traditional OTU picking methods



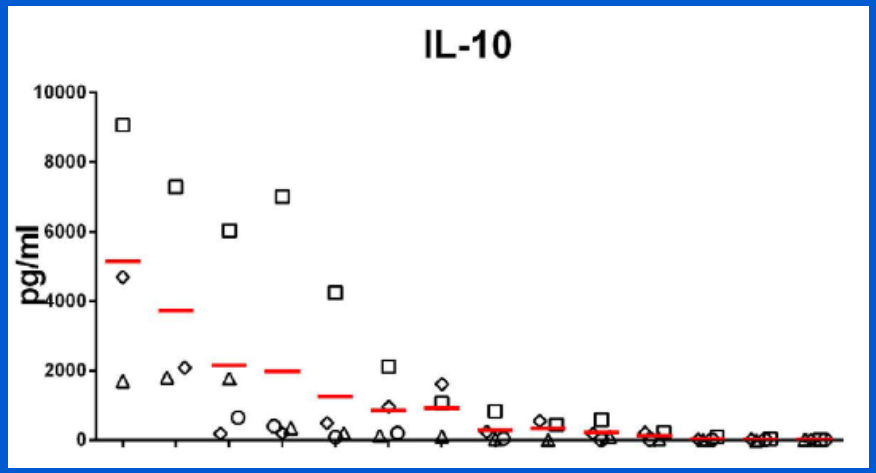
At first blush, results from the DADA2 analysis were similar





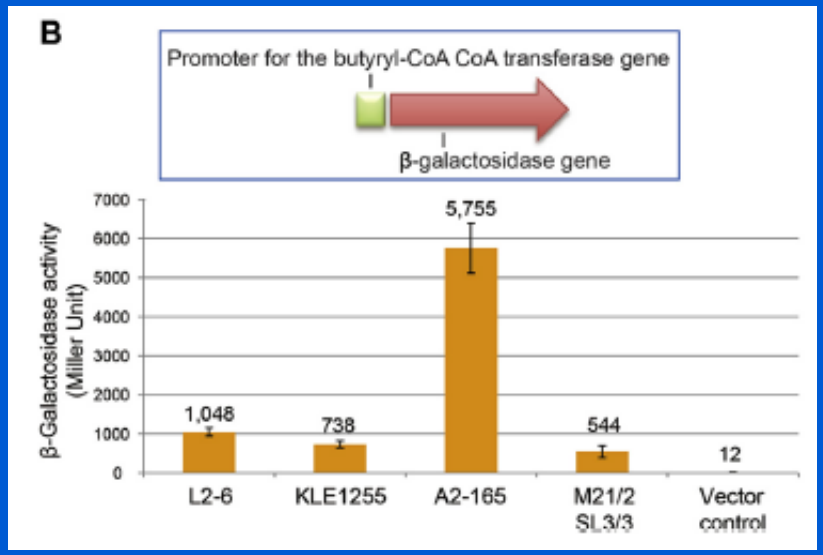
There are differences within strains of *F. praustnizii*

IL-10 production from hDC



Rossi, *Sci Rep* 2016;6:18507

Butyryl-CoA transferase gene promoter activity



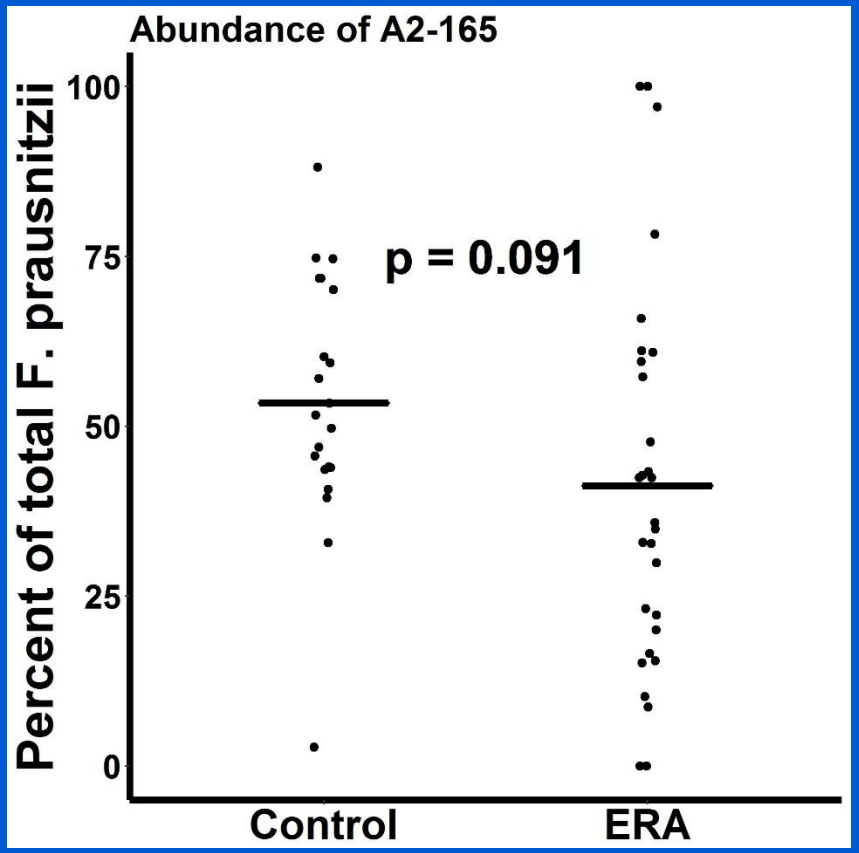
Song, *JACI* 2016;137:852

23 sequence variants matching *F. prausnitzii* were detected

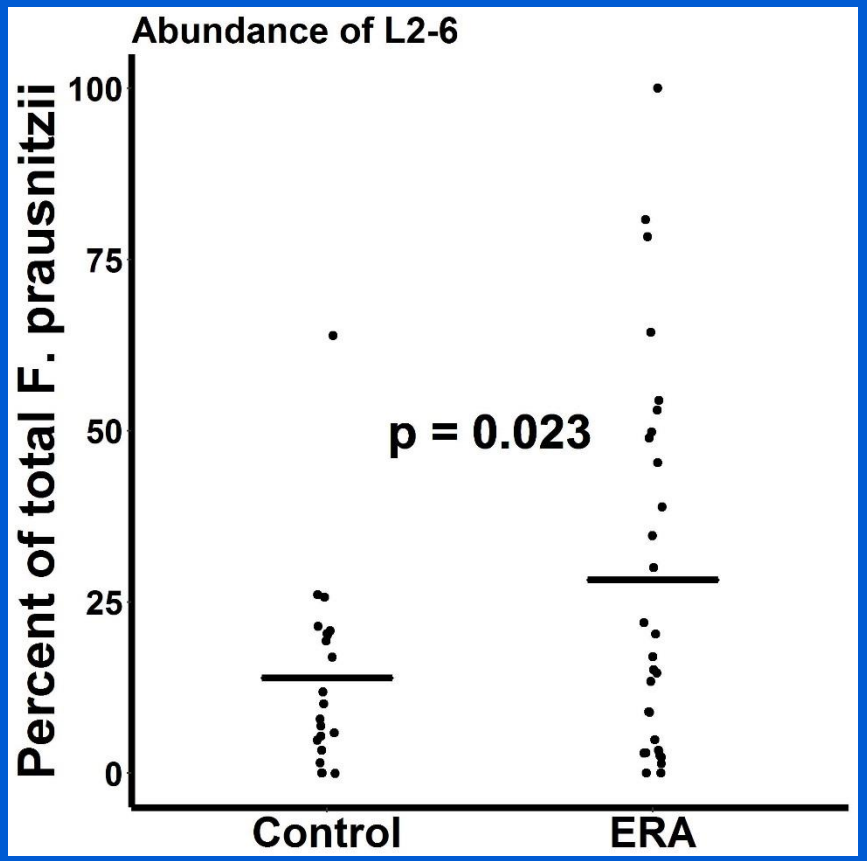
- I downloaded the 16S sequences for A2-165 and L2/6
- Ran BLAST of the 23 SVs against these two sequences
- One SV was a 100% match (253 / 253) to A2-165
- Another SV was a 99% match (252 / 253) to L2/6

Distribution of FP strains differs between patients and controls

A2-165 ("good")



L2/6 (less "good")



Whole genome sequencing

- Shotgun sequencing of all the DNA present in a sample
- May not include viral particles
- Will include human contaminant DNA

Removal of host DNA sequences

- **Not required with 16S analysis**
  - **Host DNA should not be amplified**
- **Contamination can occur with WGS**
  - **Variable with fecal microbes**
  - **High likelihood with other habitats**

Options for host DNA removal

- Reference database of host DNA
  - Filter out alignments
  - BLAST or Bowtie2 / BWA
- Reference database of microbes
  - Include sequences that align with dominant bacteria
  - Output will be limited to these bacteria

Assembly

- Most packages not designed for microbiota
  - Hundreds of species
- Unclear if even required

Options for taxonomic assessment

- **Metaphlan[1]**
  - **Assigns taxonomy based upon marker genes**
  - **Second version includes 17K reference genomes**
- **Alignment-based**
- **Composition-based: bacteria have unique sequence features**
  - **GC content**
  - **Nucleotide repeats**
  - **Codon usage**

[1]Truong *Nature Methods* 2015;10:902

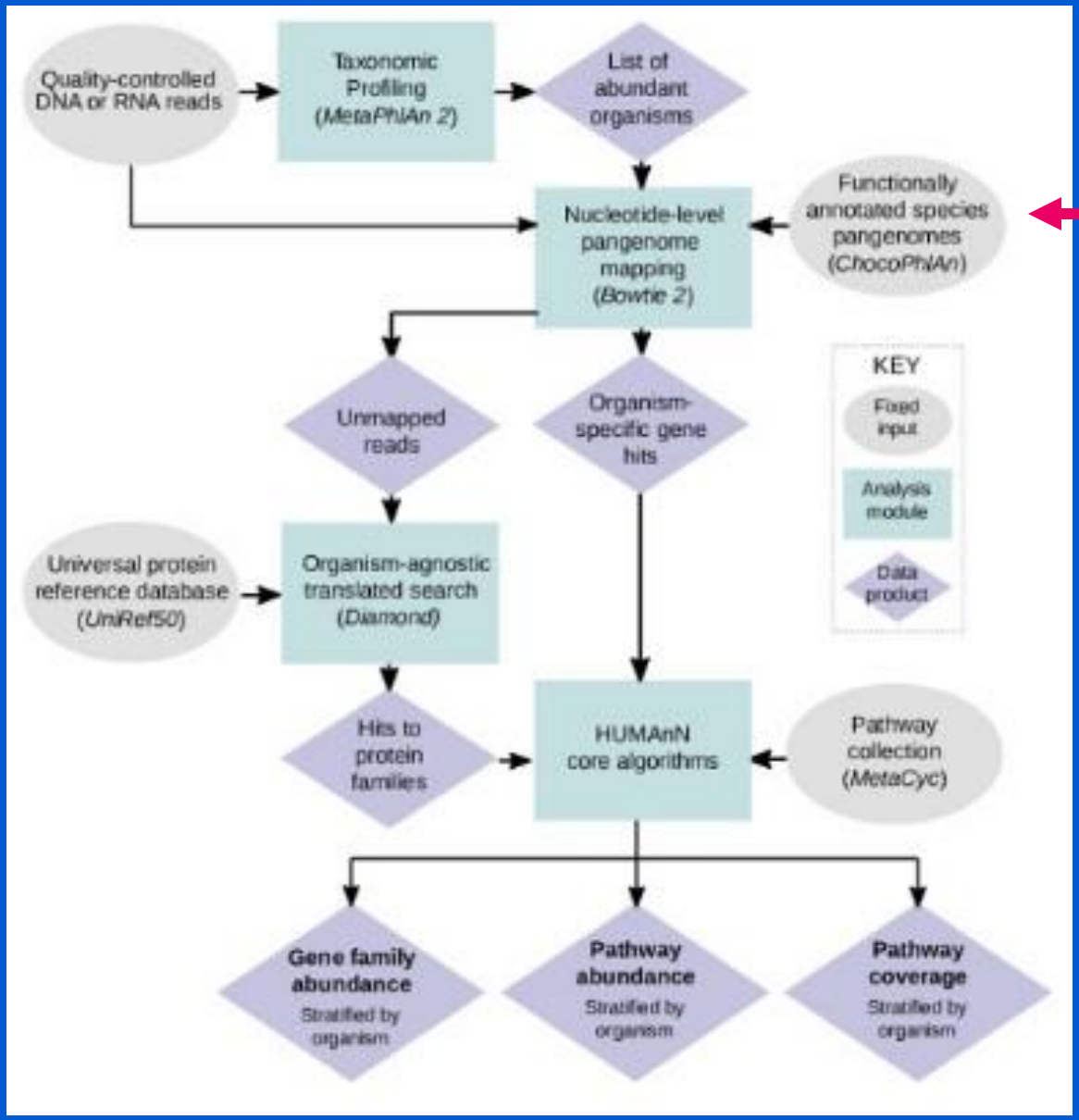
Functional annotation

- **Probably the most straightforward approach is with HUMAnN2**
- **“What are the microbes in my community of interest capable of doing?”**

[1]Abubucker *Plos Comput Biol* 2012;8:e1002358

http://huttenhower.sph.harvard.edu/humann2

HUMAnN2 workflow

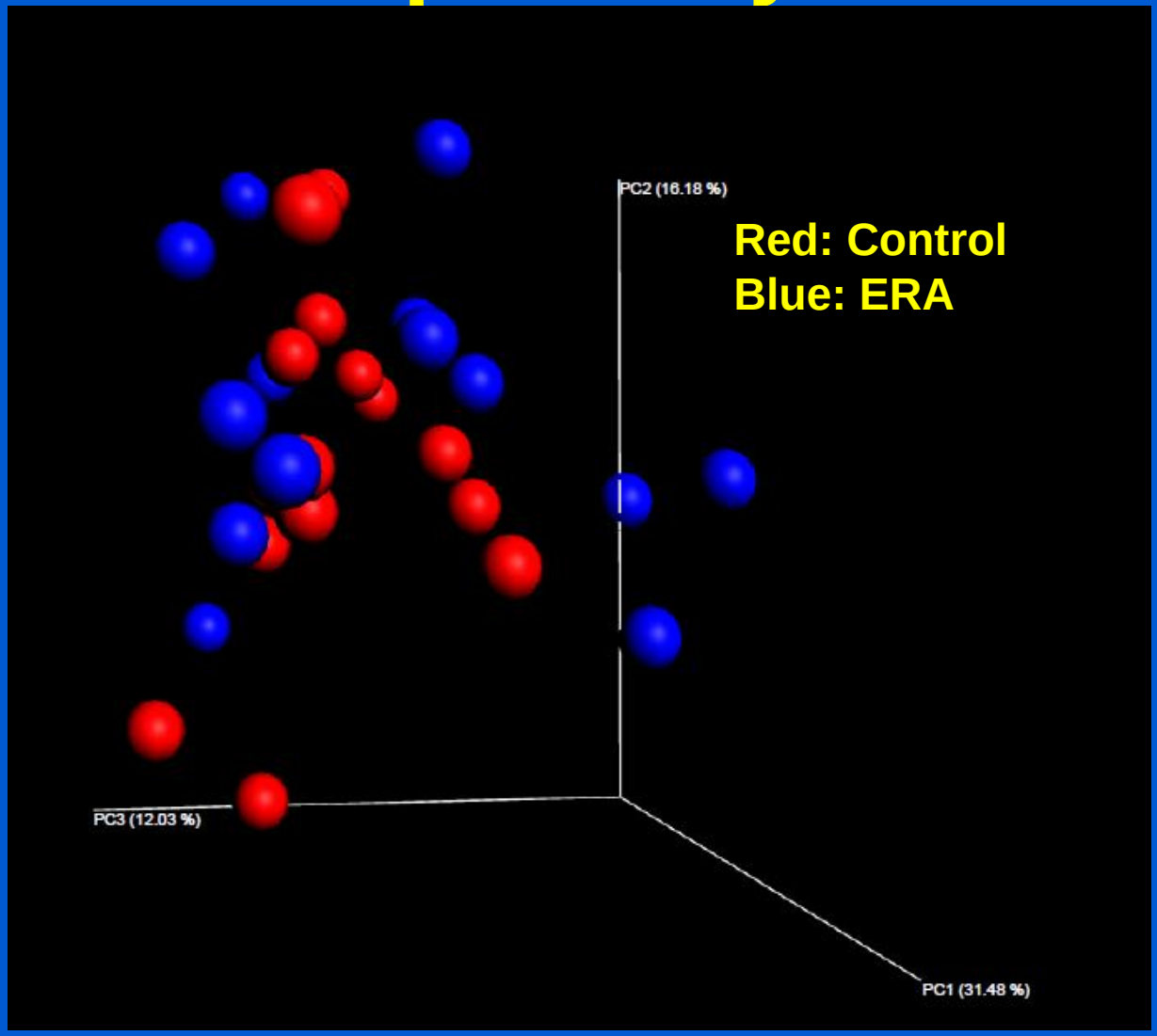


NCBI genomes

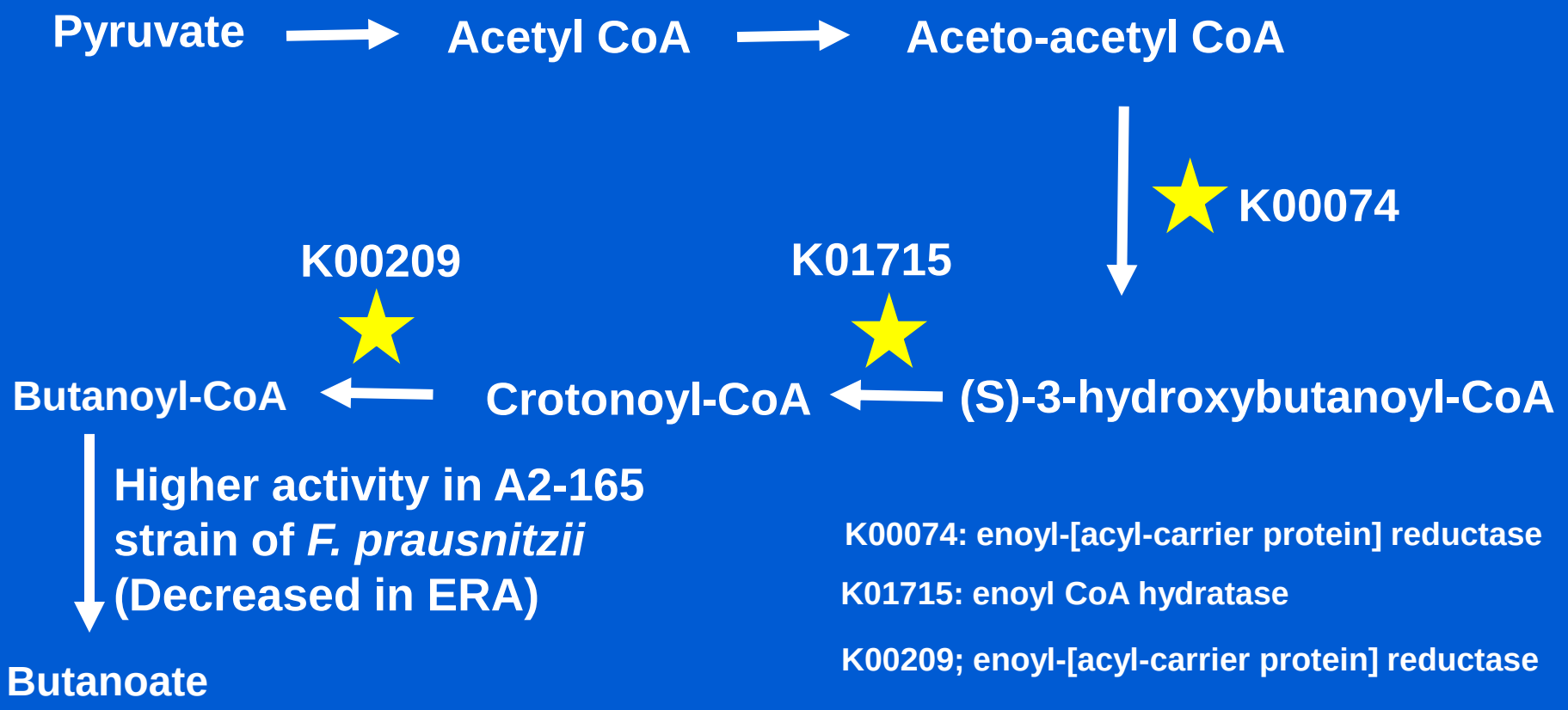
Performed whole genome sequencing of fecal microbiota from ERA patients and controls

- 14 ERA patients and 14 healthy controls
- Subset of newly diagnosed patients used for 16S analysis
- Custom quality control pipeline
- Analysis with HUMAnN2

No obvious global differences at the pathway level



Butanoate metabolism



K00074: enoyl-[acyl-carrier protein] reductase

K01715: enoyl CoA hydratase

K00209; enoyl-[acyl-carrier protein] reductase

Kanehisa *Nucleic Acids Res* 2016;44:D457

Summary

- **Study confirms association of F. *prausnitzii* in children with ERA**
  - **Findings are specific to the A2-165 strain**
- **Patients with ERA may have decreased ability to make butyrate**

Outline

- Where to sample
- Sequencing
- Data analysis
- Next steps

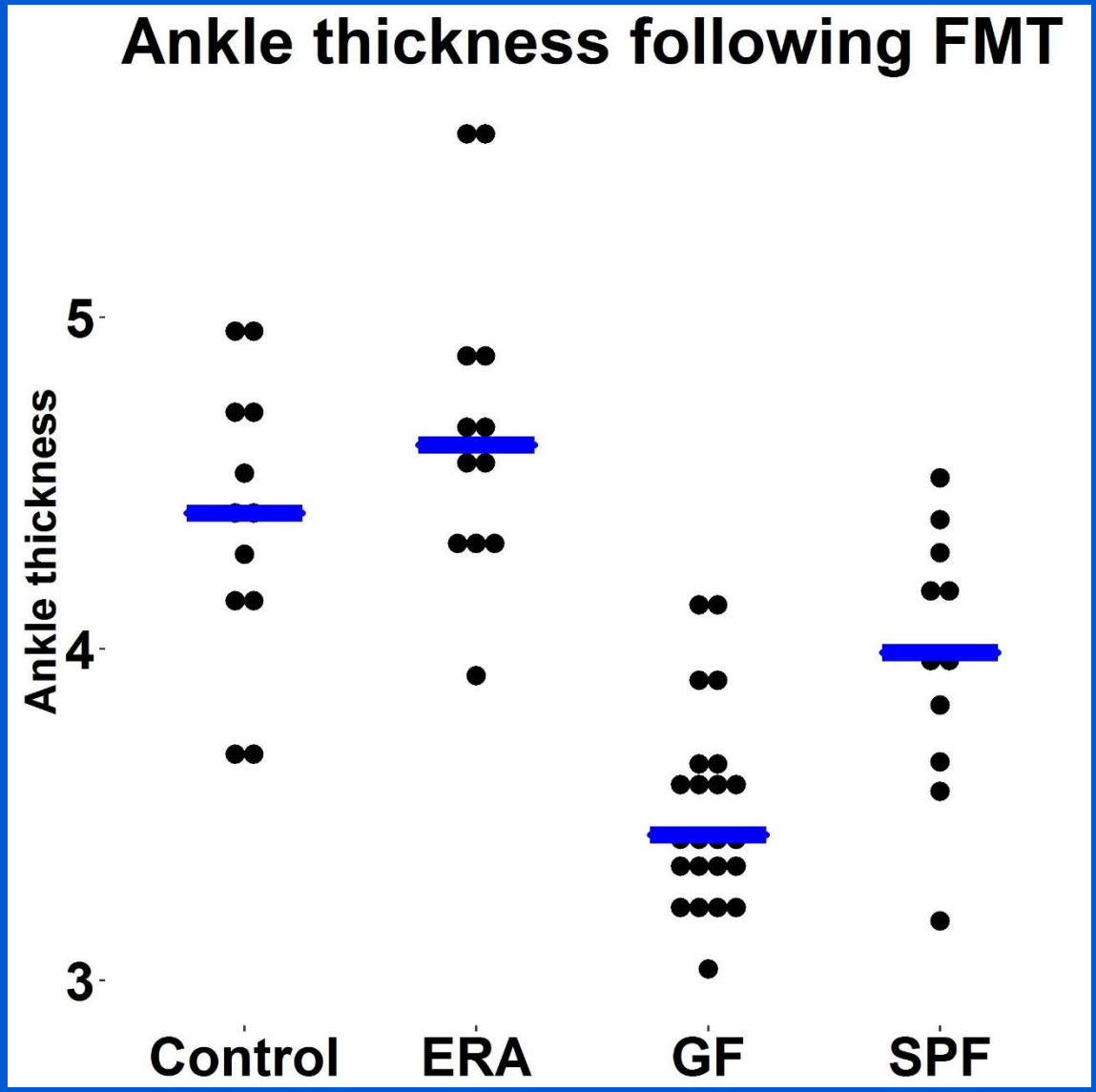
Assessing the role of the microbiota: FMT into mice

- Patient and control fecal specimens were collected in media that permits live recovery of organisms and frozen in glycerol
- Transferred to germ-free K/BxN mice
  - Arthritis model
  - Dependent on microbiota
  - Rapid onset
- Studied 3 – 4 weeks after transfer

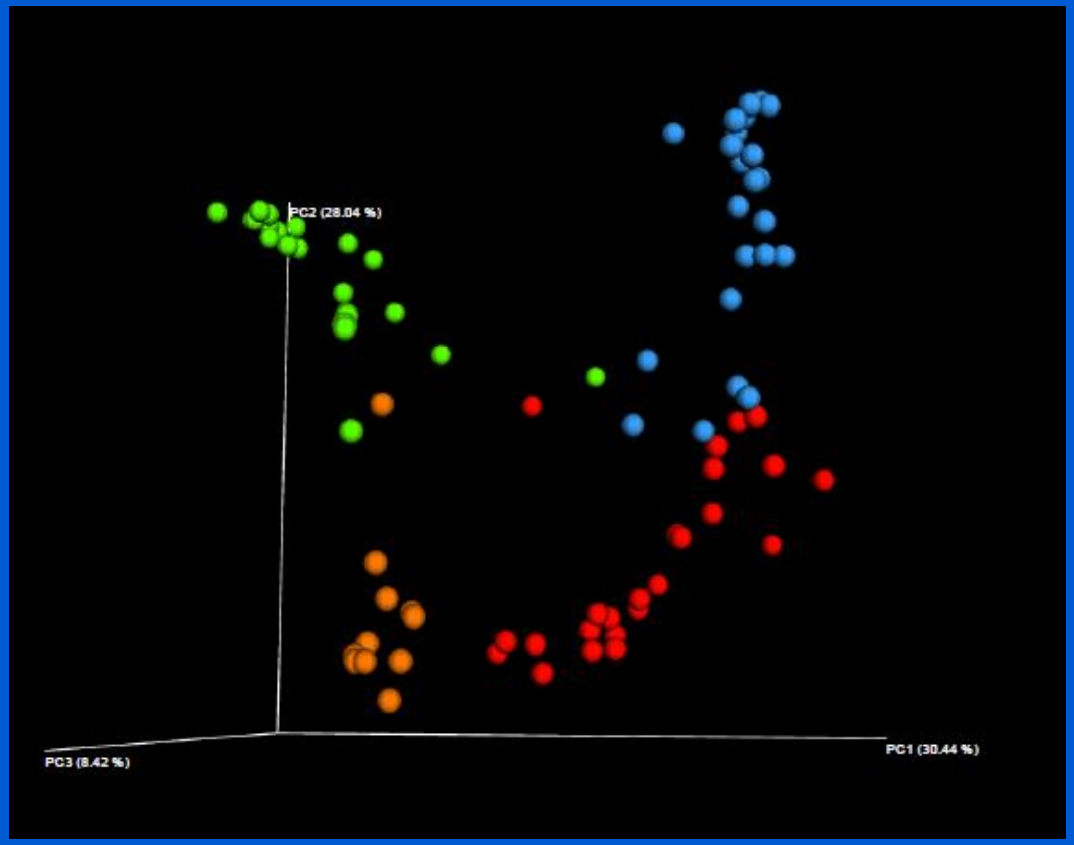
Groups

- Human donors
  - ERA, n = 12
  - Controls, n = 12
- Mouse recipients
  - ERA, n = 12
  - Controls, n = 12
- Germ-free (GF)
- Specific pathogen free (SPF)
  - Transferred from GF facility to SPF facility

Ankle swelling after FMT

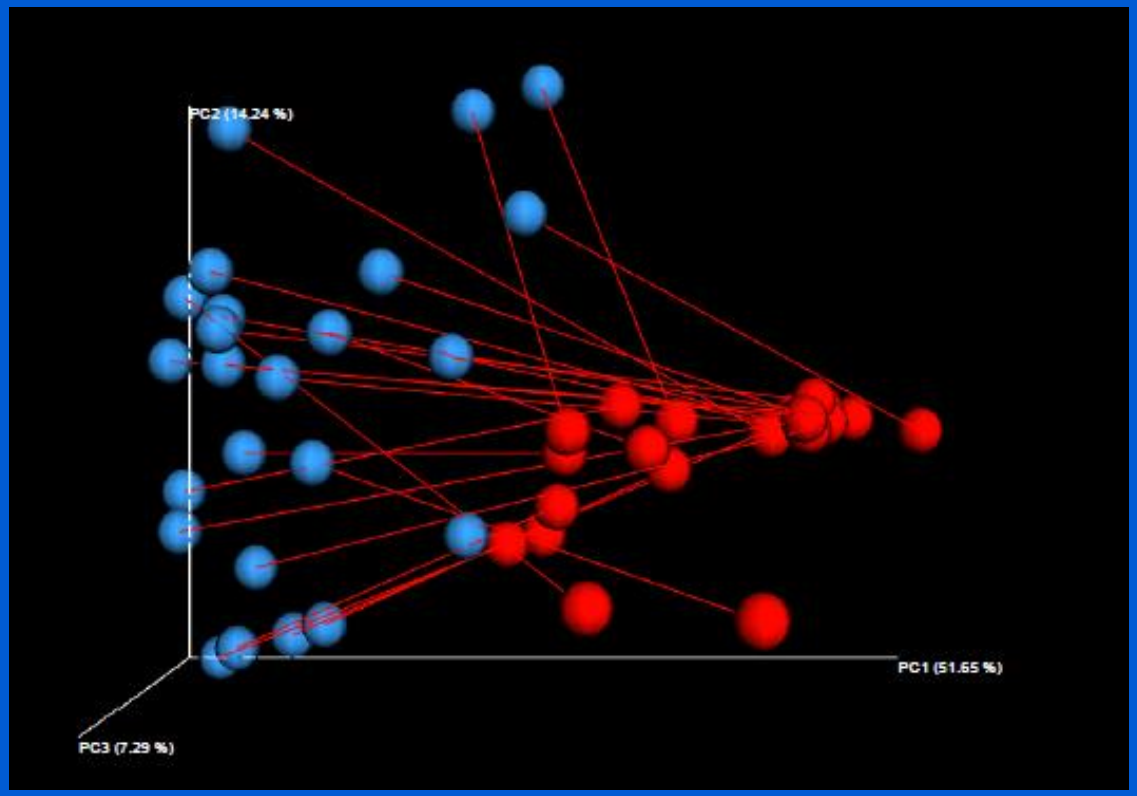


Incomplete humanization



Green: GF mice
Blue: Transplanted mice
Red: Humans
Orange: SPF mice

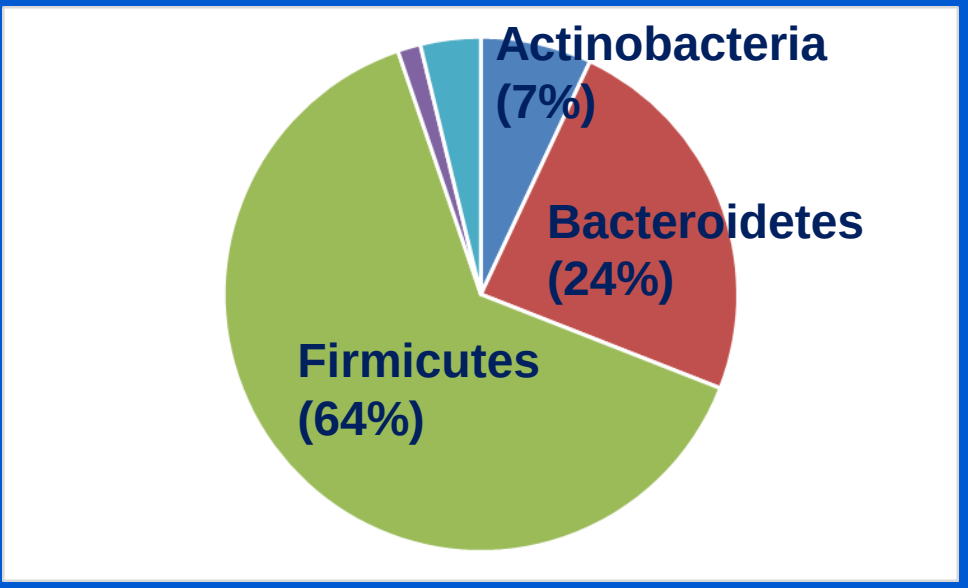
No clustering between individual patient/donor dyads



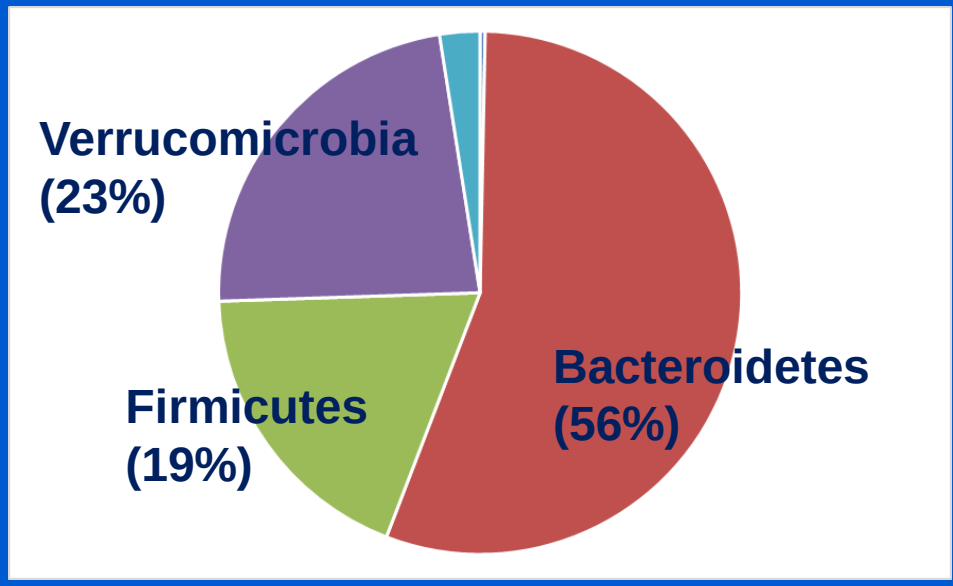
Red: Humans
Blue: Transplanted mice

Taxonomy: Phylum level

Humans

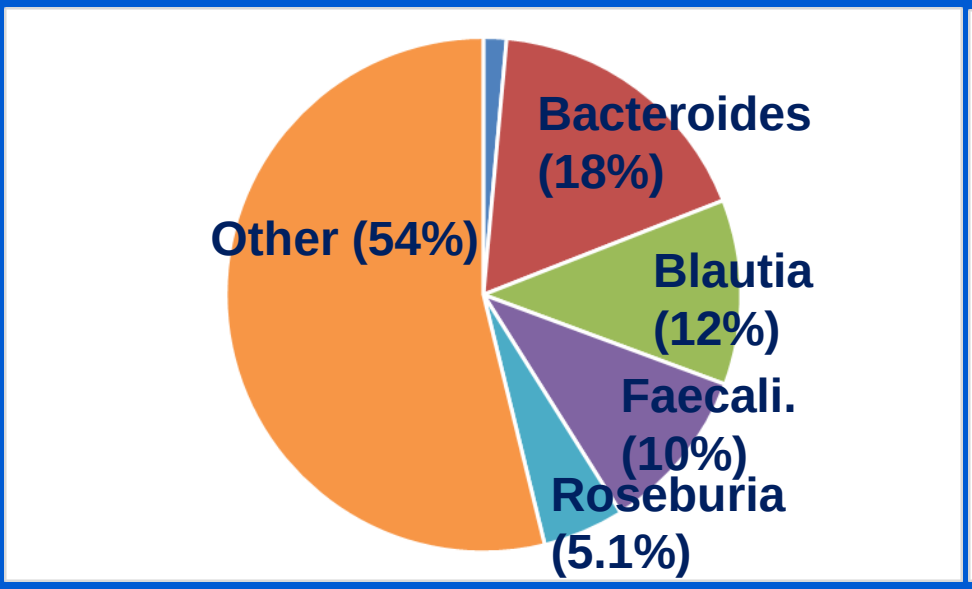


Mice

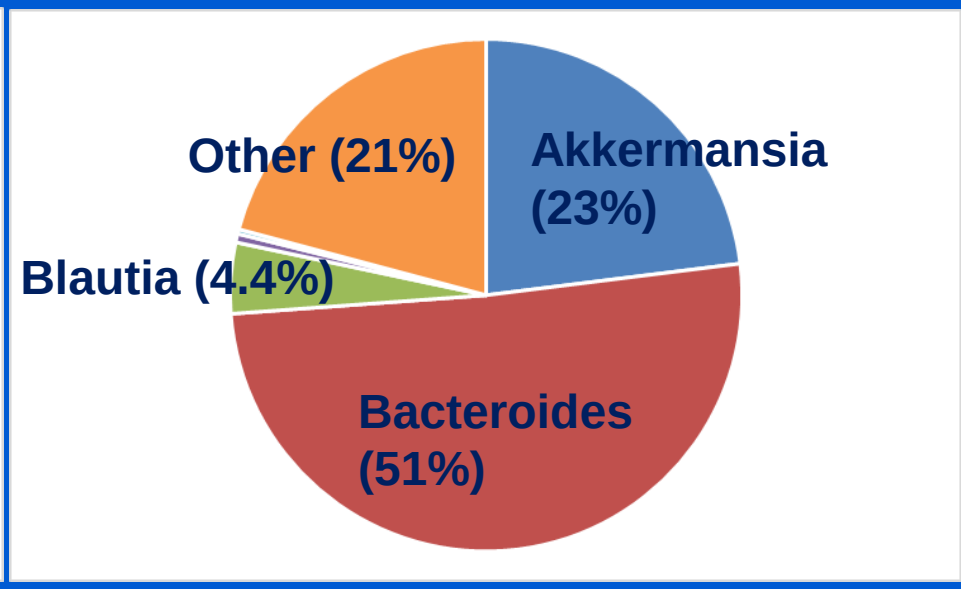


Taxonomy: Genus level

Humans



Mice



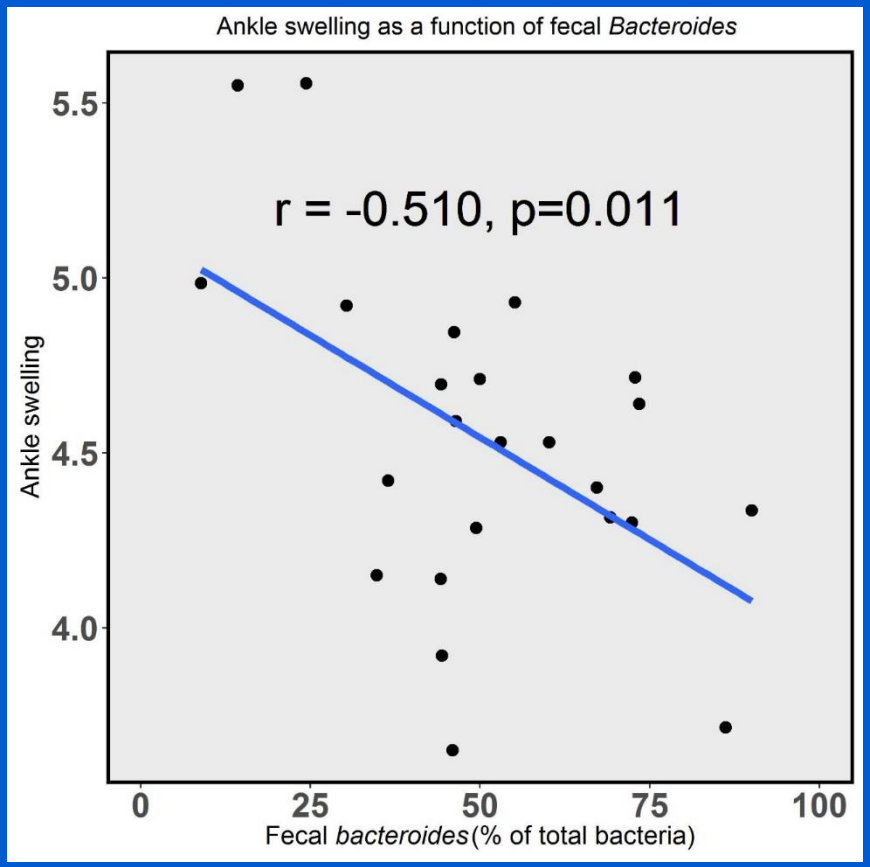
Genera present in ≥ 5% in either group

Relationship between microbiota and arthritis, among the transplanted mice

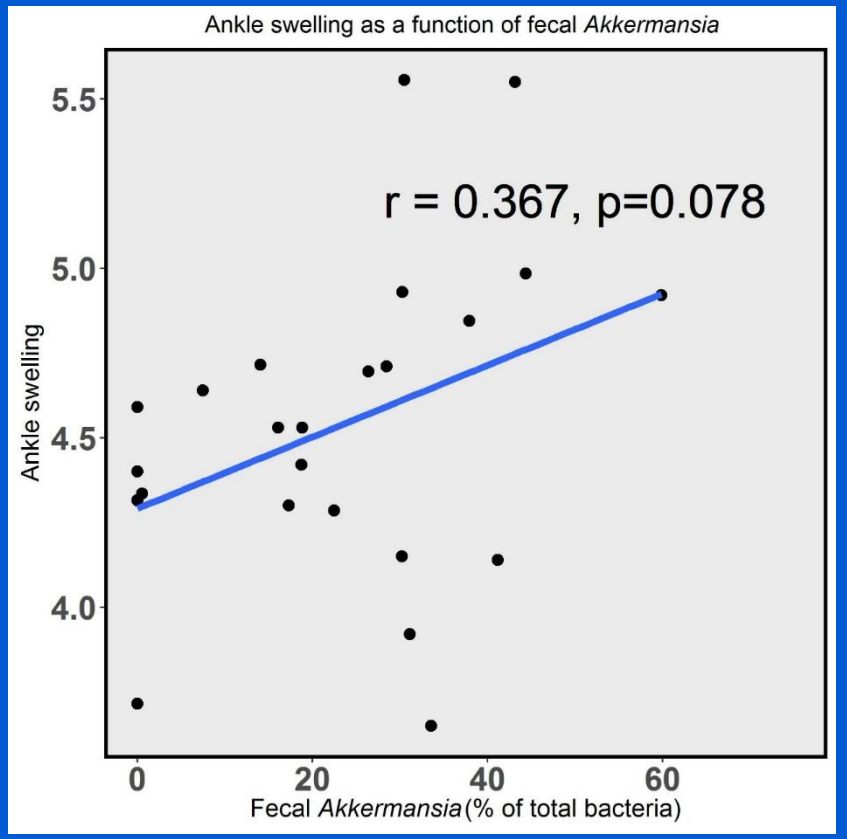
- Ran the Adonis test, which looks at whether a continuous predictor variable (in this case, ankle swelling) predicts the structure of the microbiota
- It did to some extent; $R2 = 0.185$, $p = 0.018$
- Which bacteria were driving this?

Association of common taxa with ankle swelling

Bacteroides



Akkermansia

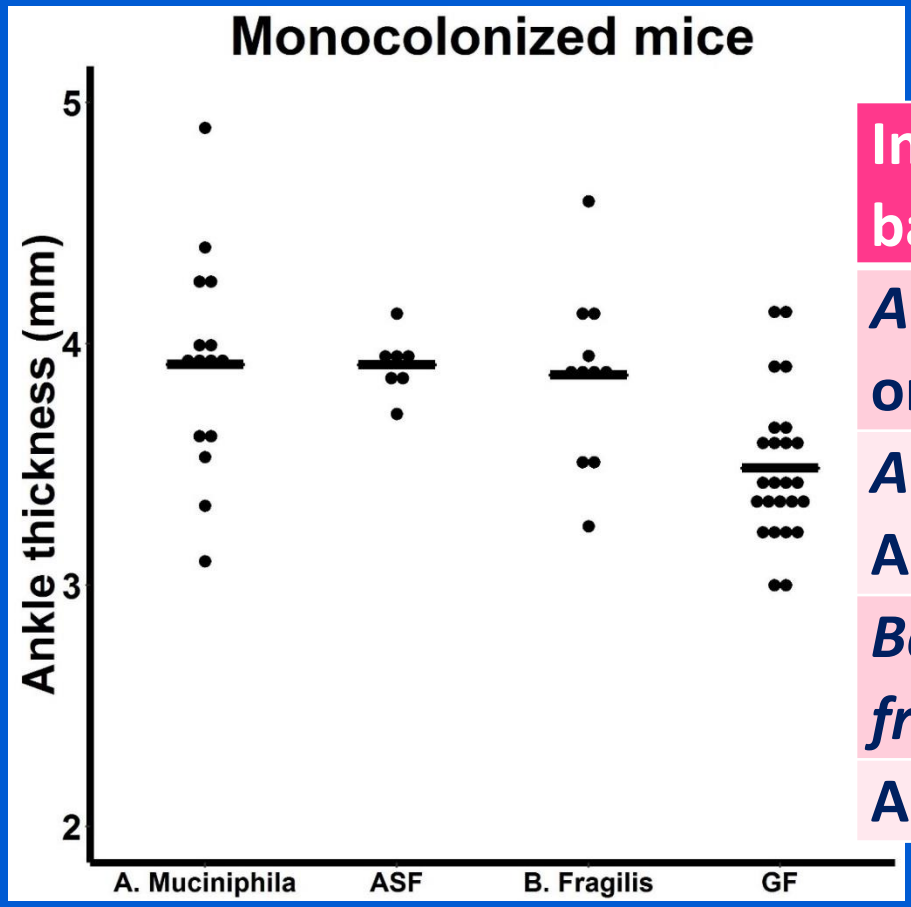


A. muciniphila may be pro- or anti-inflammatory in arthritis

- Given its name due to ability to degrade intestinal mucin
  - Potential for increased permeability
- Associated with disease in an animal model of spondyloarthritis[1]
- Decreased abundance in adults with psoriatic arthritis[2]

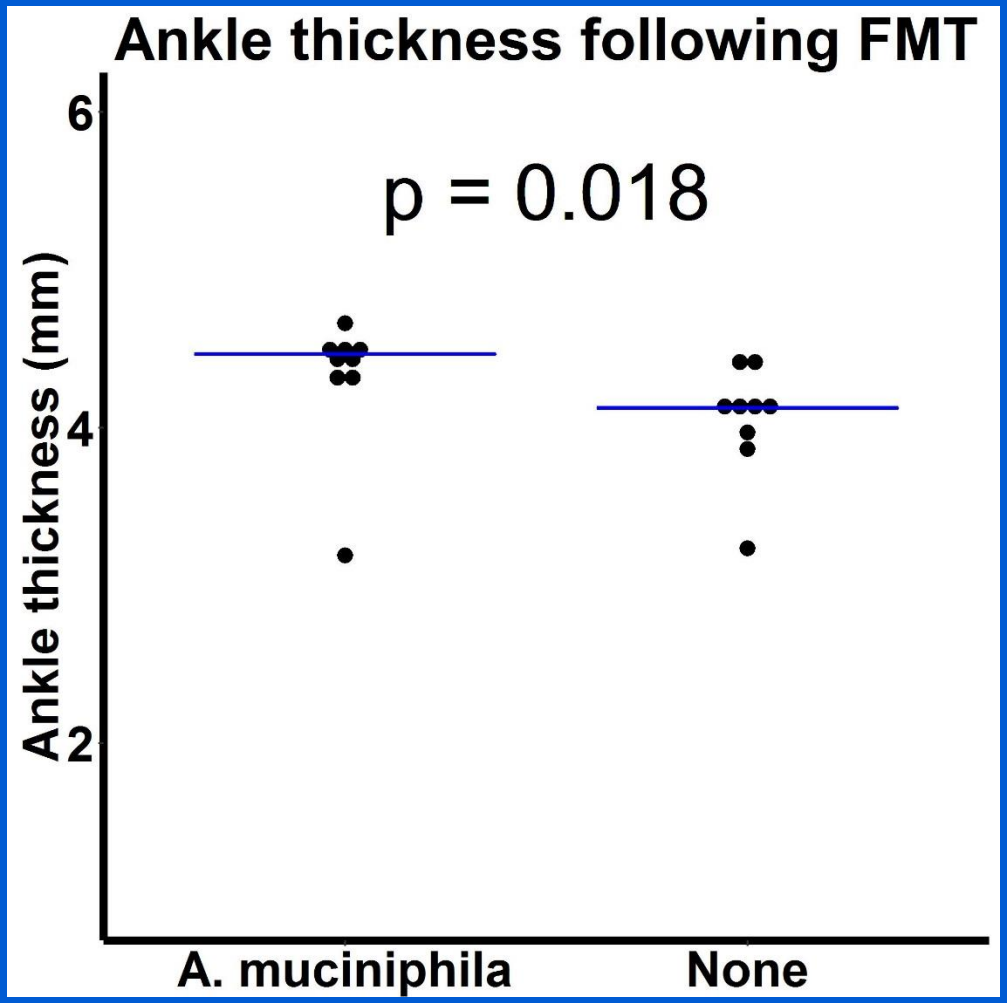
[1]Asquith, *Arth Rheum* 2016;68:2151
[2]Scher, *Arth Rheum* 2015;67:128

Monocolonization with *A. muciniphila* did not affect ankle swelling



Inoculated bacteria	Abundance of *Akkermansia* (%)
A. muciniphila only	88 ± 7.3
A. muciniphila + ASF	39 ± 14
Bacteroides fragilis + ASF	0.3 ± 0.5
ASF alone	0.3 ± 0.6

Adding *A. muciniphila* to mice receiving normal microbiota did



Summary

- ***Akkermansia*** **may facilitate arthritis, but does not appear to be directly pathogenic**
- **May relate to ability of this organism to thrive on intestinal mucin**
- **In progress: studies to evaluate for increased invasiveness of other organisms in mice receiving *Akkermansia***

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