What is the role of the microbiome in chronic airways disease?

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TAIWAN SOCIETY OF PULMONARY AND CRITICAL CARE MEDICINE ANNUAL MEETING

Images from the cystic fiboris foundation, Yarza et al Nature Rev Mic 2014

Conflicts of Interest

I have no conflicts of interest to disclose.

Agenda

1. Why should I (a pulmonologist) care about the microbiome?

2. What is the microbiome?
   (How do you do microbiome research?)

3. Is there a lung microbiome?
   (I thought the lungs were sterile?)

4. How is the microbiome relevant to chronic airways disease?

Agenda

1. Why should I (a pulmonologist) care about the microbiome?

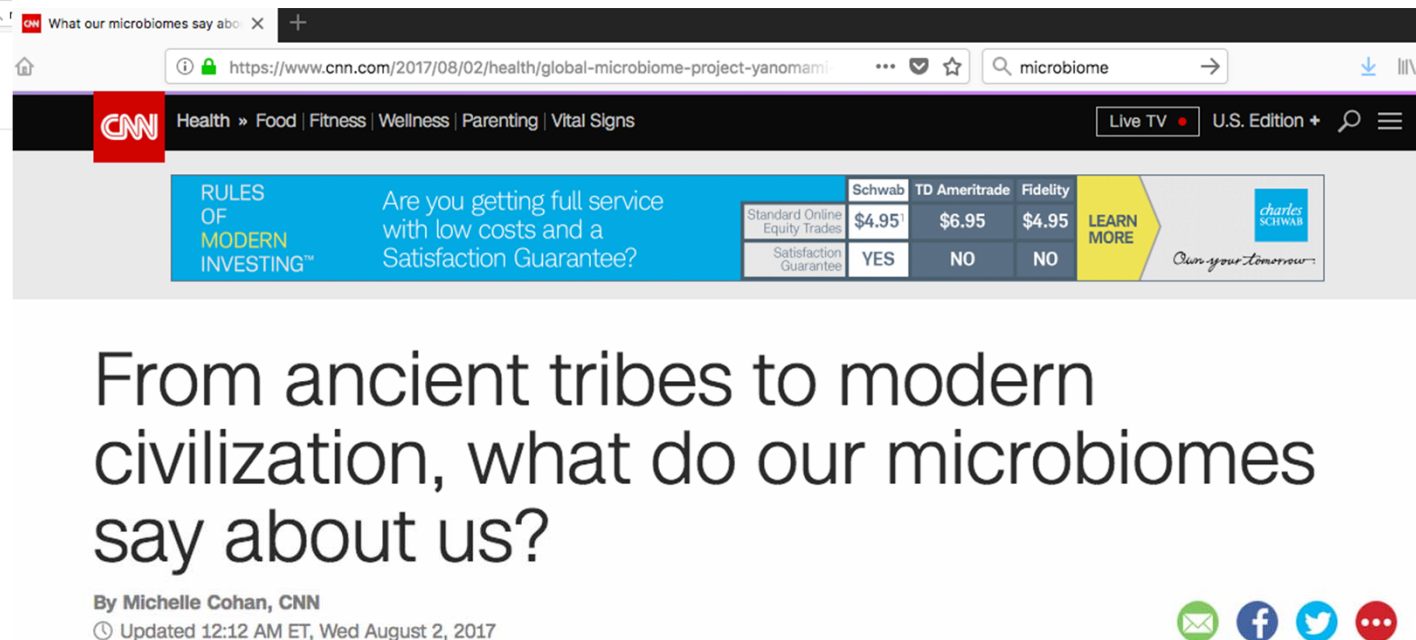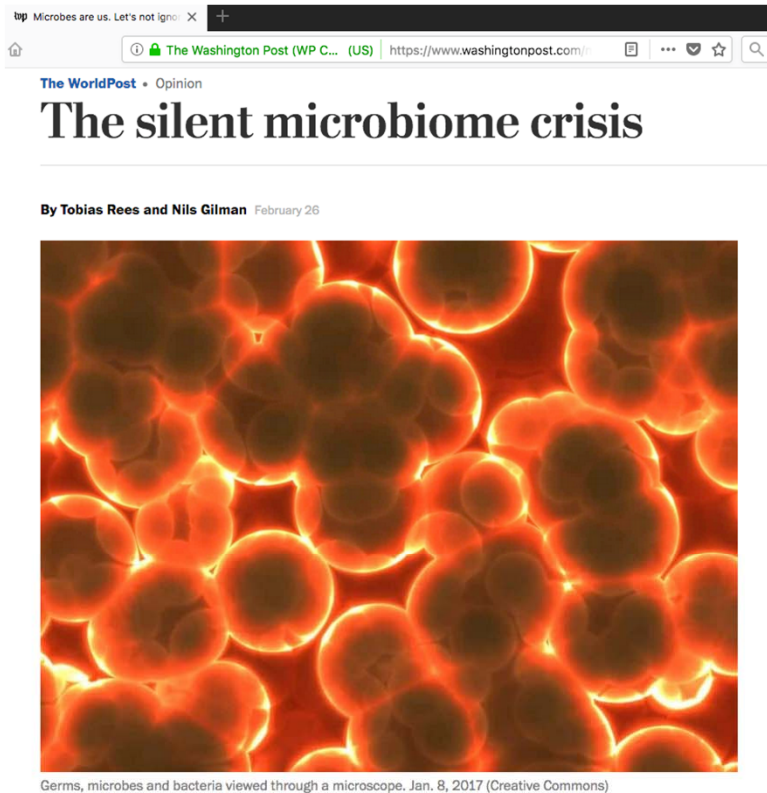2. What is the microbiome?
   (What does microbiome research look like)

3. Is there a lung microbiome?
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Everybody is talking about the microbiome

The WorldPost • Opinion

The silent microbiome crisis

By Tobias Rees and Nils Gilman February 26

Germs, microbes and bacteria viewed through a microscope. Jan. 8, 2017 (Creative Commons)

From ancient tribes to modern civilization, what do our microbiomes say about us?

By Michelle Cohan, CNN
Updated 12:12 AM ET, Wed August 2, 2017

Everybody is talking about the microbiome

Pubmed citations for "microbiome[tiab]"
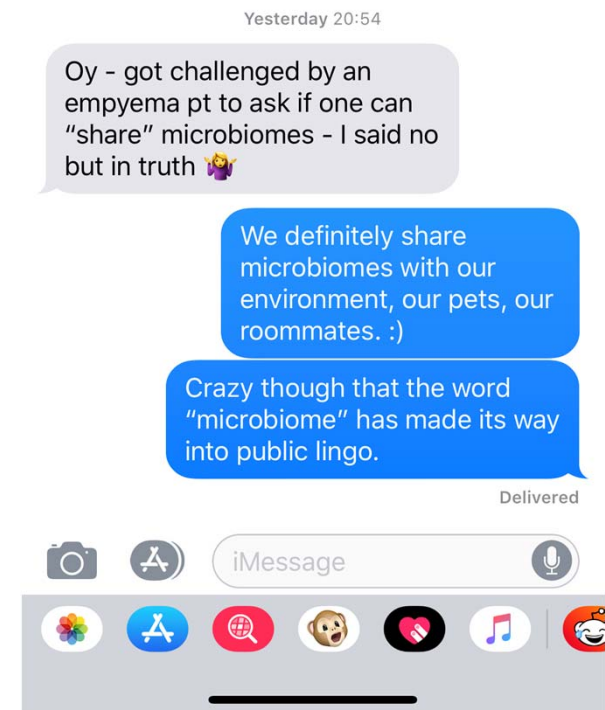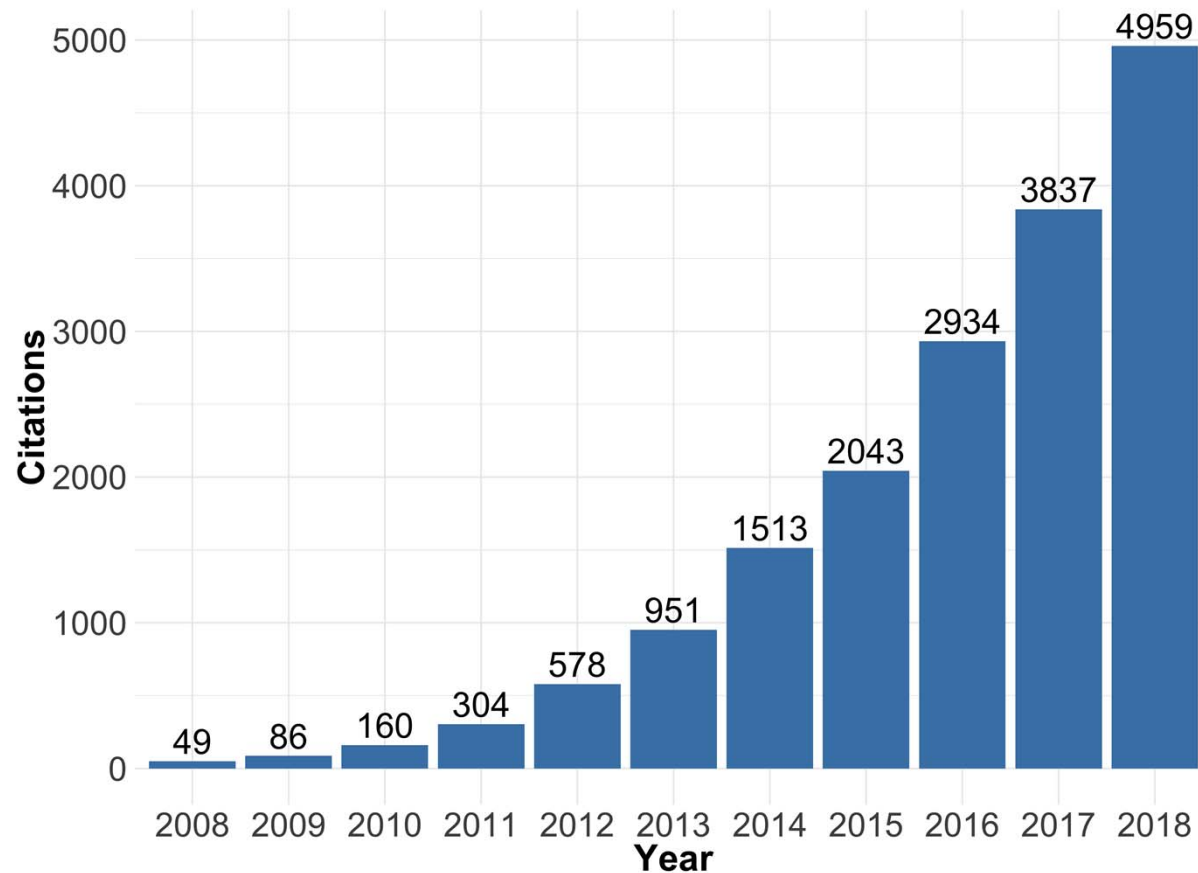
Fecal transplantations impact health (sometimes in surprising ways)

First performed in humans in 1958 (earlier in animals)

Refractory C. difficile: cure rate 91%[1]

Neonatal sepsis: RCT of probiotic + prebiotic: RR 0.60 [0.48 – 0.74] [2]

Autism: Pilot studies encouraging[3], trials ongoing

Parkinson's disease: human fecal transplant → germ-free mouse model[4]

1. Mello et al Am J Gast 2011. PMID: 22675283
2. Panigrahi et al. Nature 2017. PMID:28813414
3. Kang et al. Microbiome 2017. PMID:28122648
4. Sampson et al. Cell 2016. PMID:27912057

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How do we measure microbes?

"Alive and culturable"

Culture

"How much"
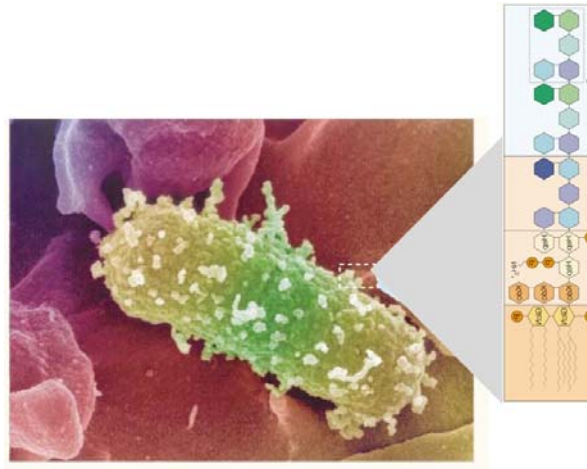
Microbial fragments + qPCR
- Endotoxin
- Peptidoglycan
- 1,3, beta D Glucan

"Who is there"
"What are they doing"

Sequencing
- Amplicon (16S, 18S, ITS)
- Shotgun

Beutler et al, Nature Rev Imm 2003 PMID:18293536,
Yarza et al Nature Rev Mic 2014 PMID:25118885
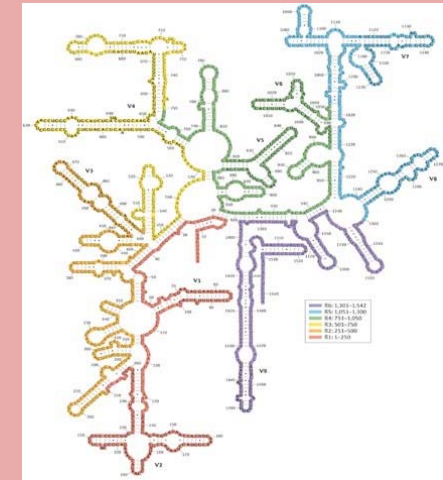
Amplicon sequencing (16S, 18S, ITS)

Microbial community sample

Extract DNA

Amplify DNA
Sequence DNA

Group sequences
• OTU or ASV

OTU1 GATACAGAGATGCAT
GATACAGTGATGCAT
GATACAGTAGATGCA
GATACAGTAGATGCA

OTU2 TACCAGATTTACATA
TACCAGATTACATAC
TACCAGATTACATAC

OTU3 CAGGTAGGATAATA
CAGGTAGGATAATA
CAGGTAGGATAATTA
CAGGTAGGAAATA

OTU4 GATTACAGATTACA
GATTACAGATTTACA
GATTACAGAATTACA

NCBI
GreenGenes
myRDP
Silva

Taxonomy
• Match OTU/ASV to database

Who is there?

Relative abundance (taxa)   Phylogeny

Abundance

Taxa

Shotgun (metagenomics) sequencing

Microbial community sample → Extract DNA → Sequence DNA Remove human reads → Match sequence to functional databases
- KEGG
- SEED
- BLAST
→ What are they doing?

Relative abundance (gene pathways)

Compare sequence to reference genome → Who is there?

Relative abundance (taxa)   Phylogeny   SNPs

Taxa

GATTACA
GATTACA
GATTTCA
GATTTCA
GATTTCA
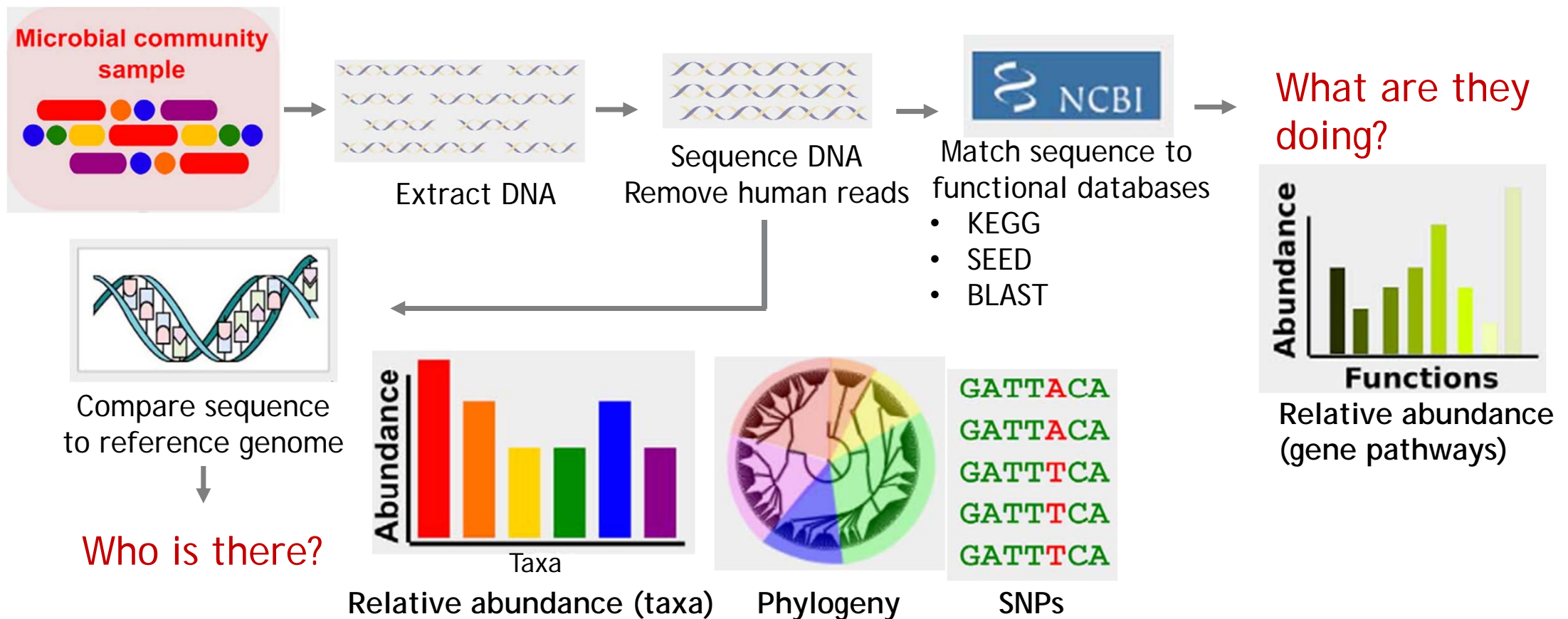
Amplicon vs Shotgun sequencing

	Amplicon (16S, 18S, ITS)	Shotgun
Cost (sequencing)	$45	$400 → $200

Taxonomy

Kingdom	*Bacteria*	*Animalia*	
Phylum	*Proteobacteria*	*Chordata*	
Class	*Gammaproteobacteria*	*Mammalia*	
Order	*Enterobacteriales*	*Carnivora*	
Family	*Enterobacteriaceae*	*Felidae*	
Genus	*Escheria*	*Felis*	
Species	*Escheria coli*	*Felis catus*	
Strain	*Escheria coli O157:H7*	-	

Felis silvestris

Felis catus

Bias (primer amplification, extraction, sequencing)

Mock community	
Species	(%)
Pseudomonas aeruginosa	12.0
Escherichia coli	12.0
Salmonella enterica	12.0
Lactobacillus fermentum	12.0
Enterococcus faecalis	12.0
Staphylococcus aureus	12.0
Listeria monocytogenes	12.0
Bacillus subtilis	12.0
Cryptococcus neoformans	2.0
Saccharomyces cerevisiae	2.0
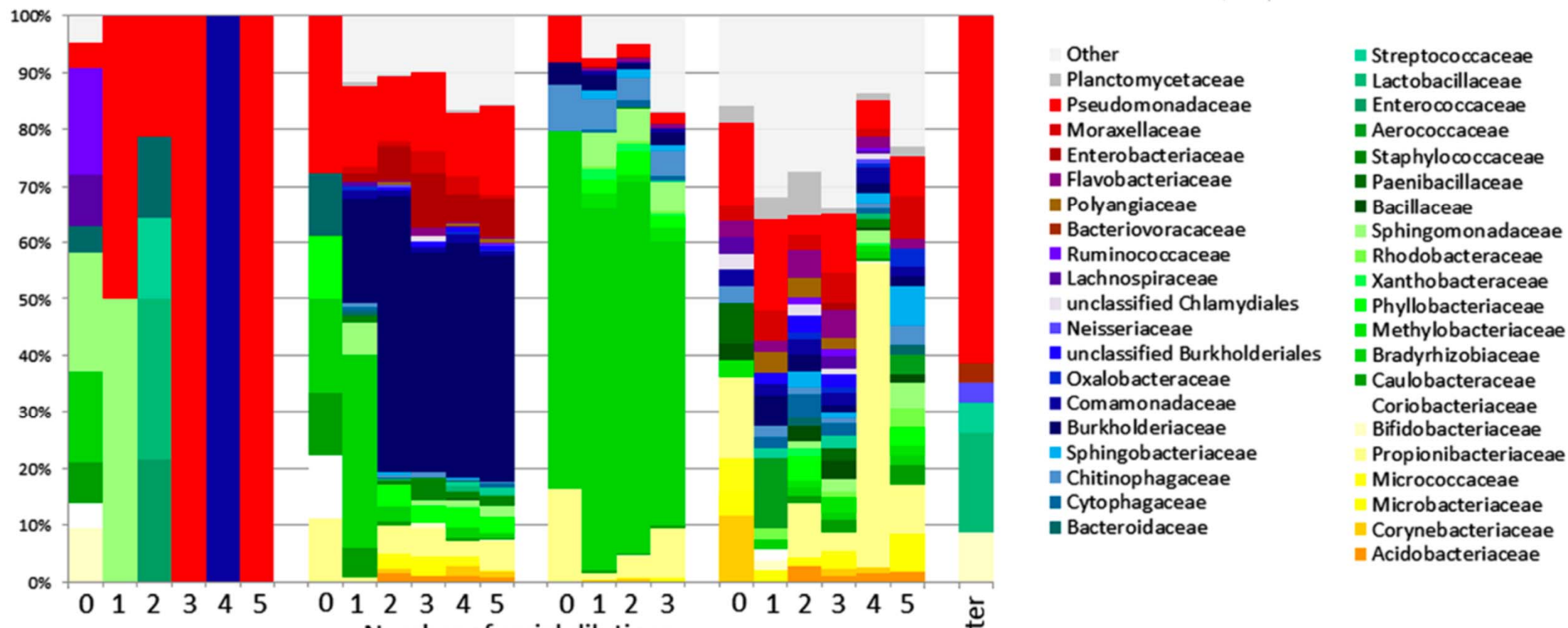
Kit Contamination

WORSE WITH:

- AMPLICON SEQUENCING
- LOW BIOMASS SAMPLES

Salter et al. BMC Biology. 2014. PMID: 25387460

Microbiome refers to entire community of microbes
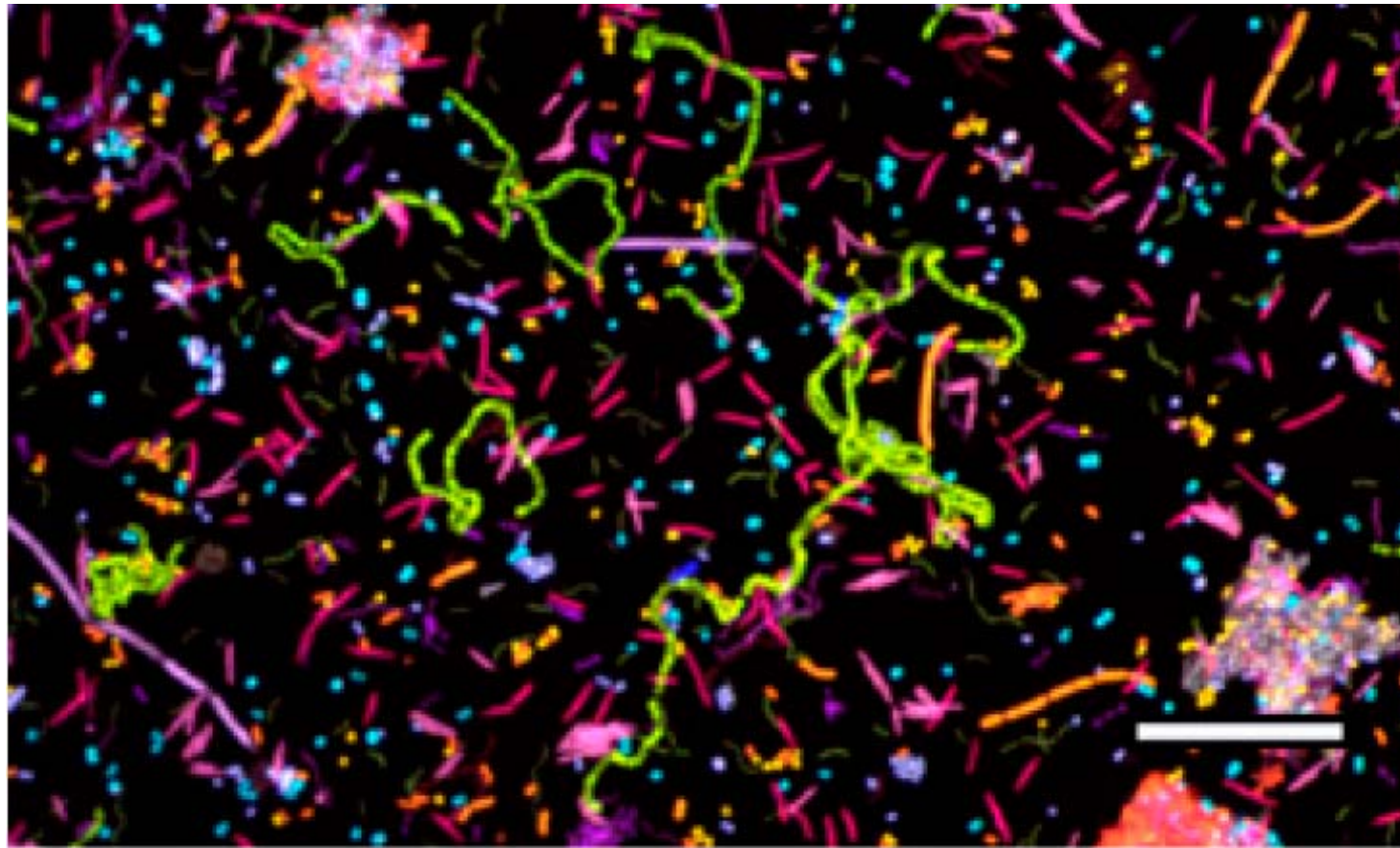
Alexa fluor 488
Alexa fluor 514
Alexa fluor 555

Rhodamine Red X
Alexa fluor 594
Alexa fluor 647

Terminology

Microbiota ("Who is there?")

- Community of microorganisms in a particular environment
- bacteria, fungi, viruses, archaea
- up to 10 microbial cells per human cell
- OTU: Operational Taxonomic Unit
- ASV: Amplicon Sequence Variant

Microbiome ("Who is there? What are they doing?")

- The combined genetic material of the microorganisms in a particular environment
- 200 microbial genes for each human gene

Terminology

Richness

- Number of species or taxa found in a sample

Evenness

- Relative distribution of species or taxa in a sample

Diversity

- α diversity: within a sample
- β diversity: differences between samples

Dysbiosis

- Imbalance in microbial ecosystem associated with disease vs health

Most microbes are beneficial if not essential

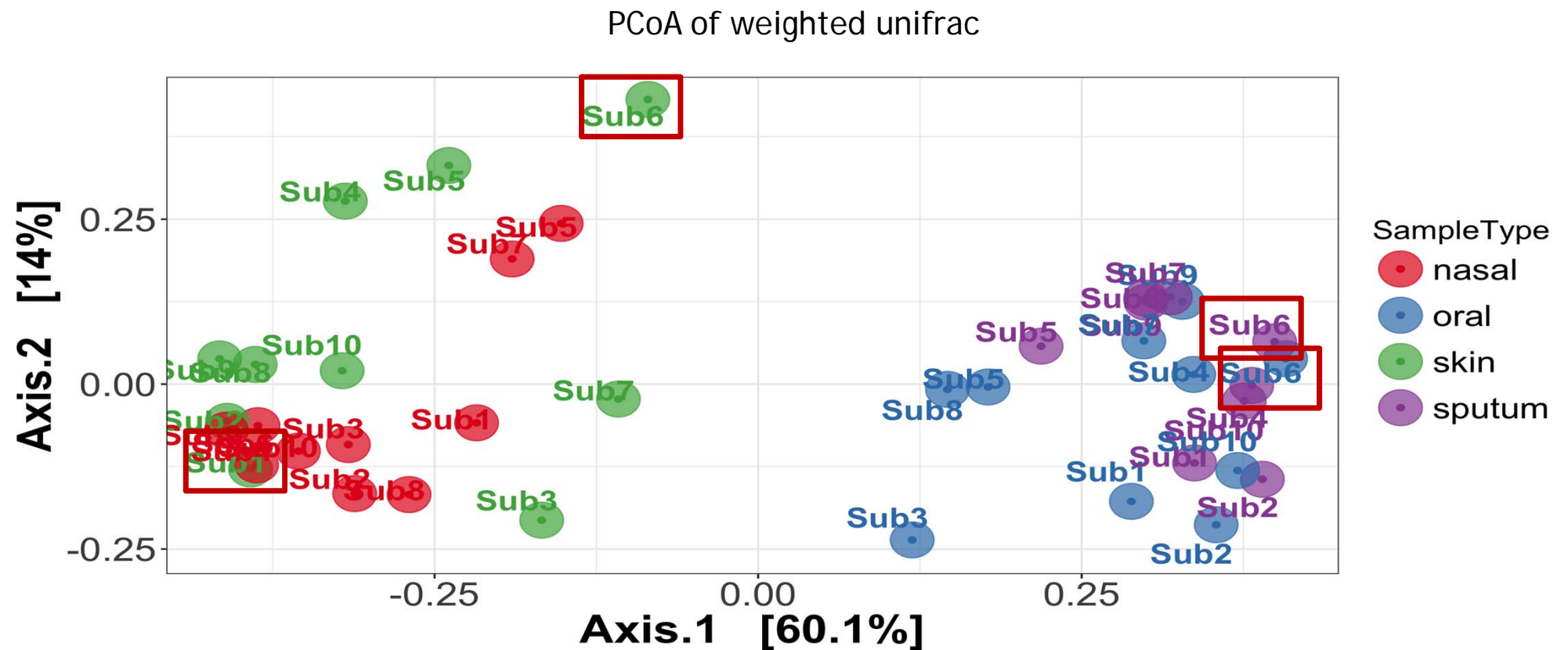# Most microbes are beneficial if not essential

Human microbiota determined by environment (body site)

PCoA of weighted unifrac

Indoor microbiota also determined by environment too (room type)

Animal Room

Cage Wash Area

Dirty	Middle	Set Up (Clean)
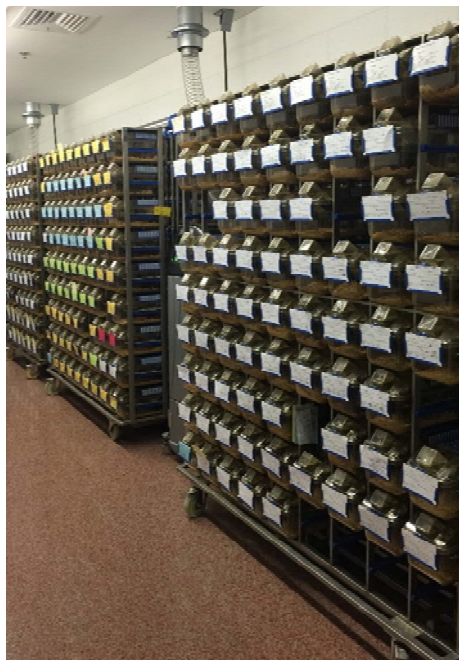
Indoor microbiota also determined by environment (room type)

PCoA of weighted unifrac
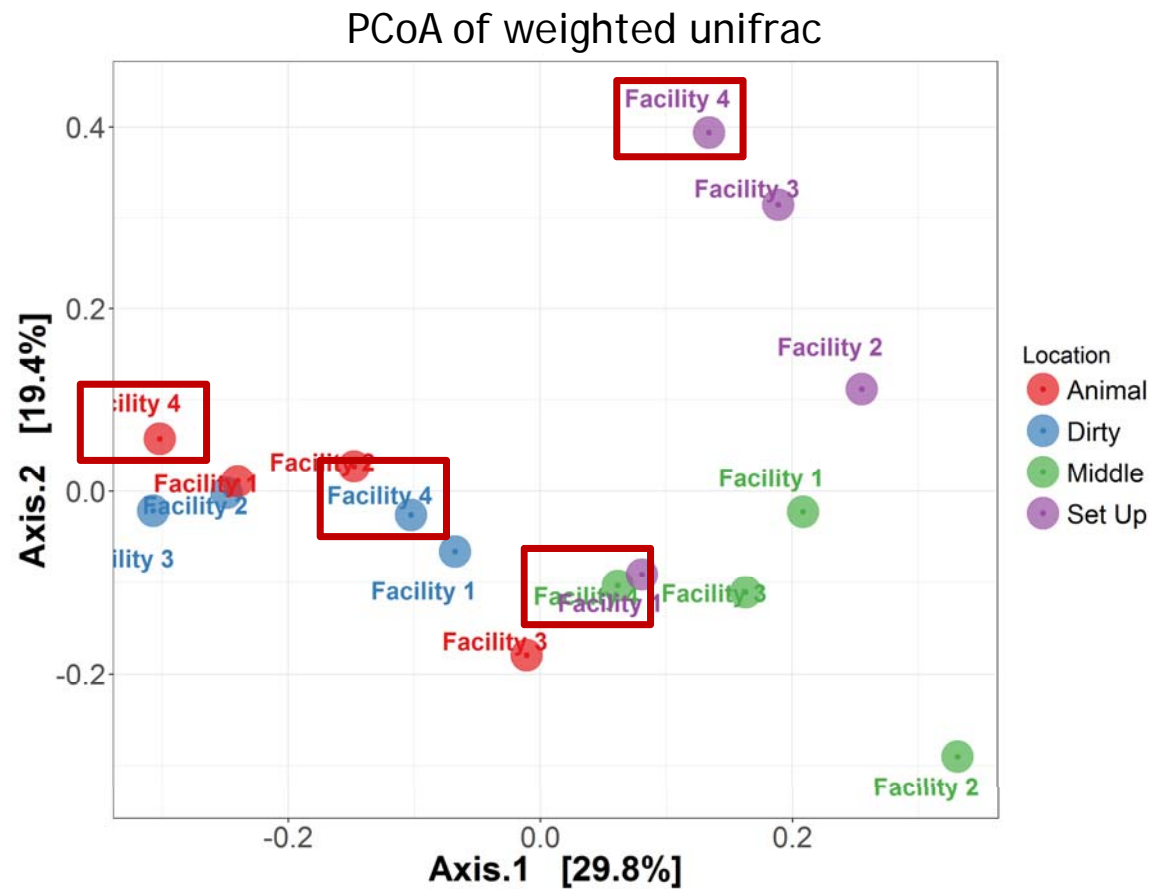
Taxonomy different, but function conserved

a Phyla

Legend: Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fusobacteria, Tenericutes, Spirochaetes, Cyanobacteria, Verrucomicrobia, TM7
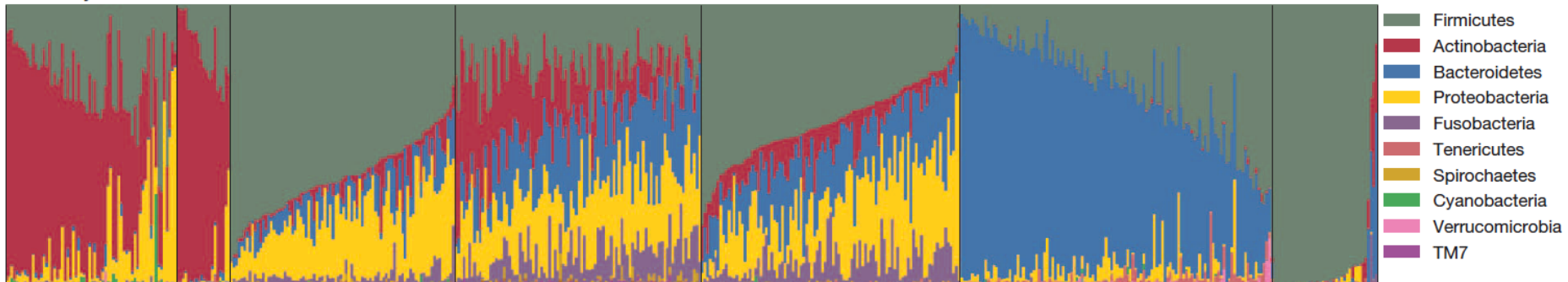
Anterior nares | RC | Buccal mucosa | Supragingival plaque | Tongue dorsum | Stool | Posterior fornix

Influences on the human microbiome
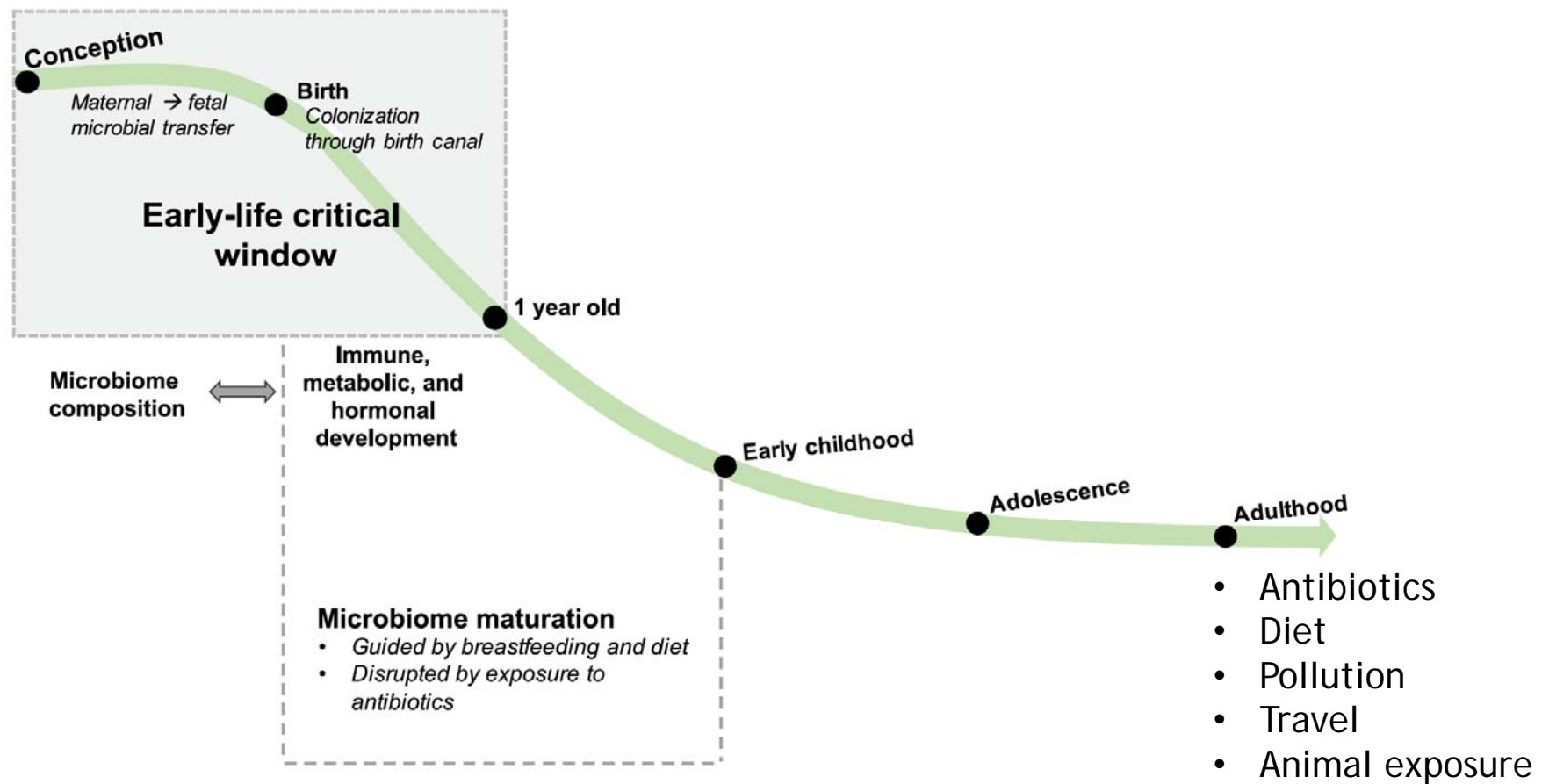
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First lung microbiome study shows differences between controls, asthma, and COPD
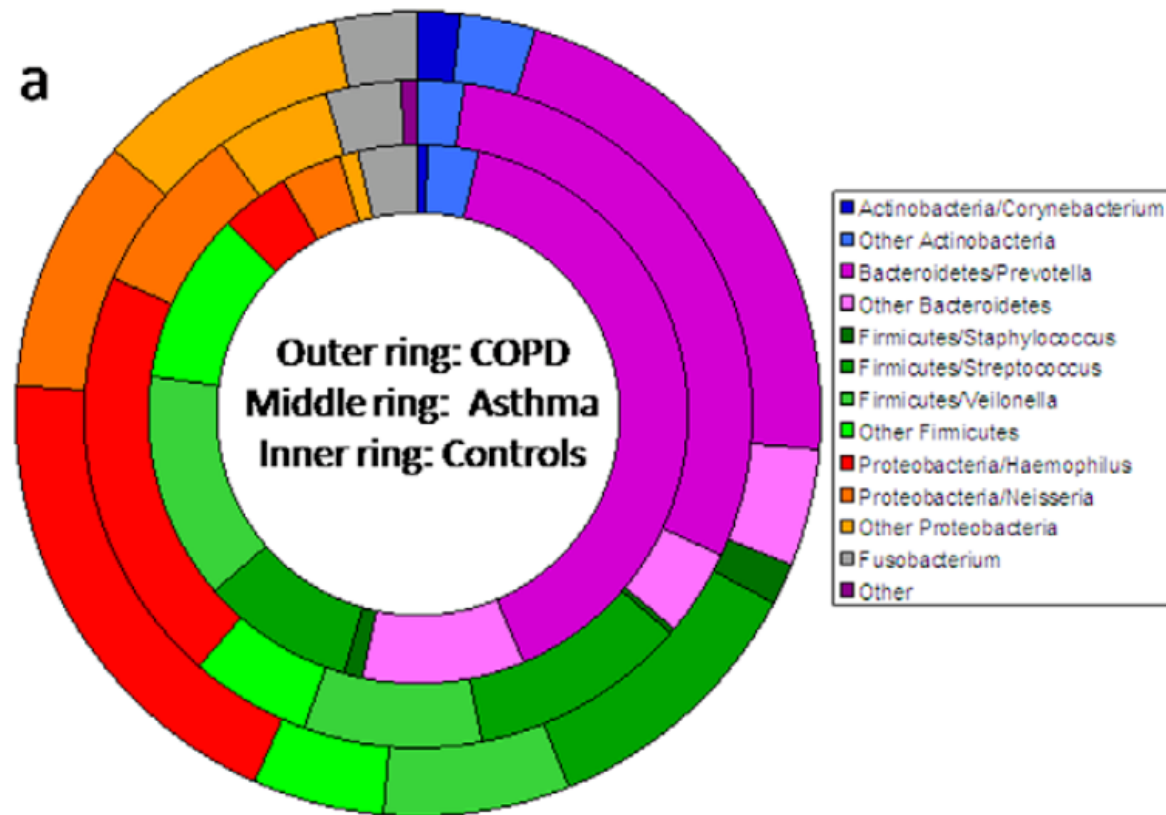
Surgical lung biopsy addresses contamination from mouth -> qPCR demonstrates bacteria

Relative abundance of bacteria differs by disease

Where does the lung microbiome come from?
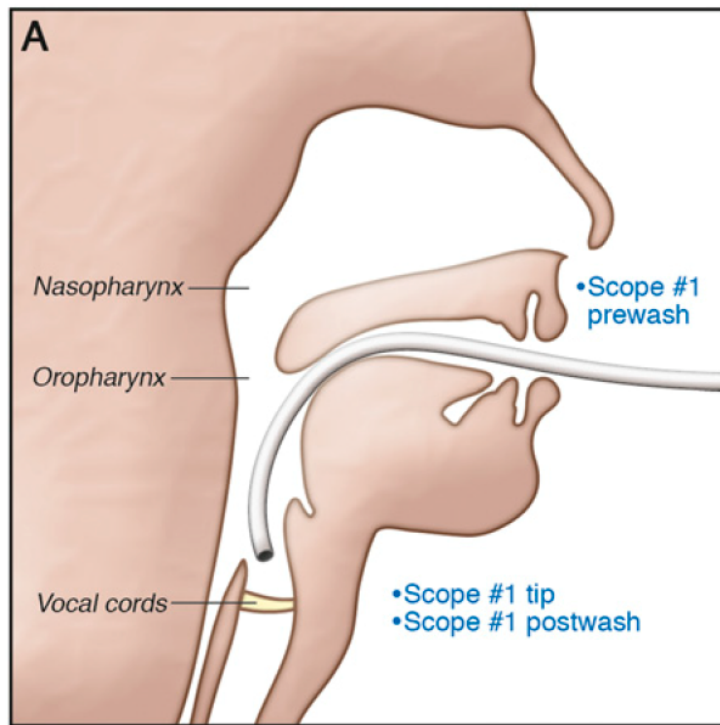
Scope 1
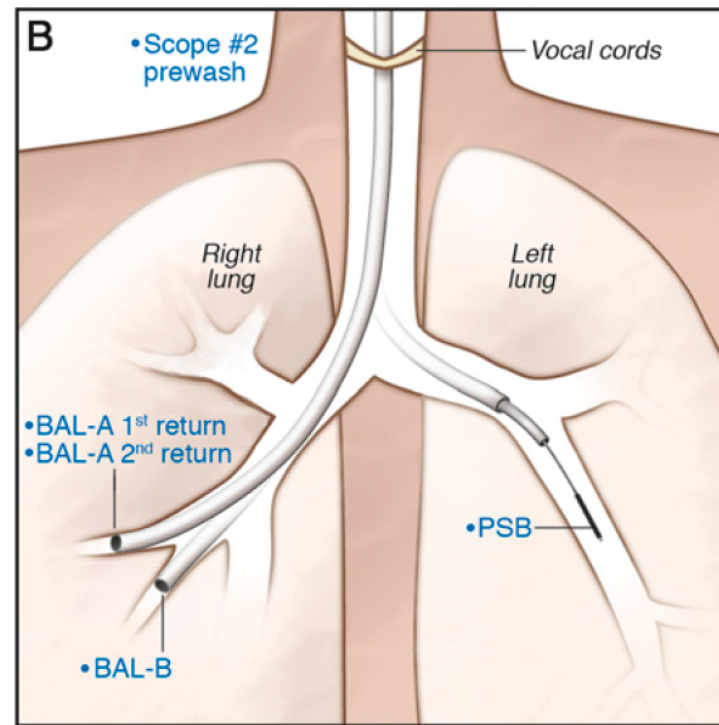
Scope 2

Lung microbiome comes from the oropharynx

	Extraction Controls	Scope 1 Pre-Wash	Scope 2 Pre-Wash	NP swab	OP swab	OW	Scope 1 Tip-Swab	Scope 1 Post-Wash	BAL A 1st Ret	BAL A 2nd Ret	BAL B	PSB
Absolute number 16S rDNA copies Mean (range)	1,135# (725-1,622)	2,093# (734-5.0E4)	1,448# (792-2.5E3)	34,223^ (4.8E3-1.5E5)	6.79E7^ (1.94E6-2.58E8)	1.93E7# (1.3E6-6.2E7)	135,637^ (9.1E3-5.3E5)	102,064# (1.7E3-4.7E5)	26,923# (4.0E3-5.8E4)	8,209# (892-2.0E4)	8,024# (1.1E3-3.9E4)	172,271^ (1.7E3-7.9E5)

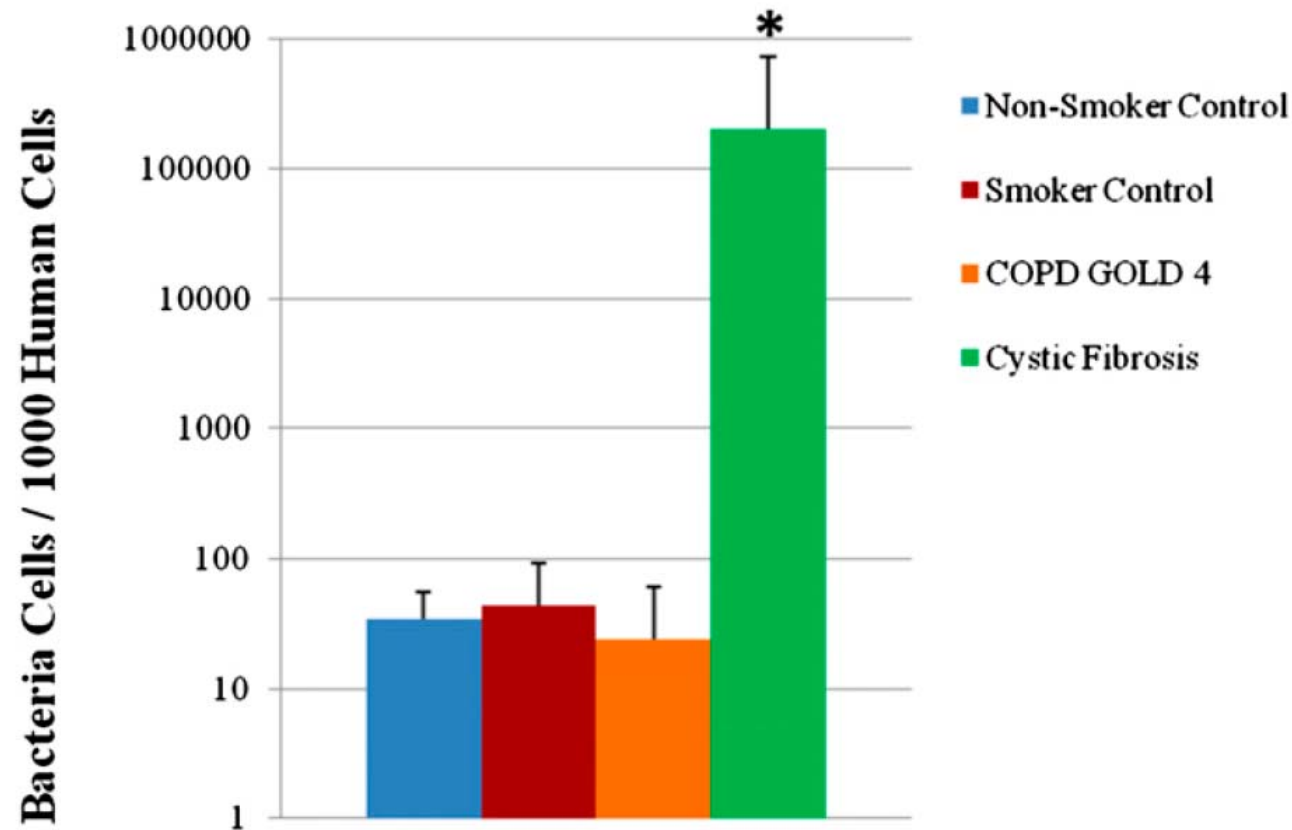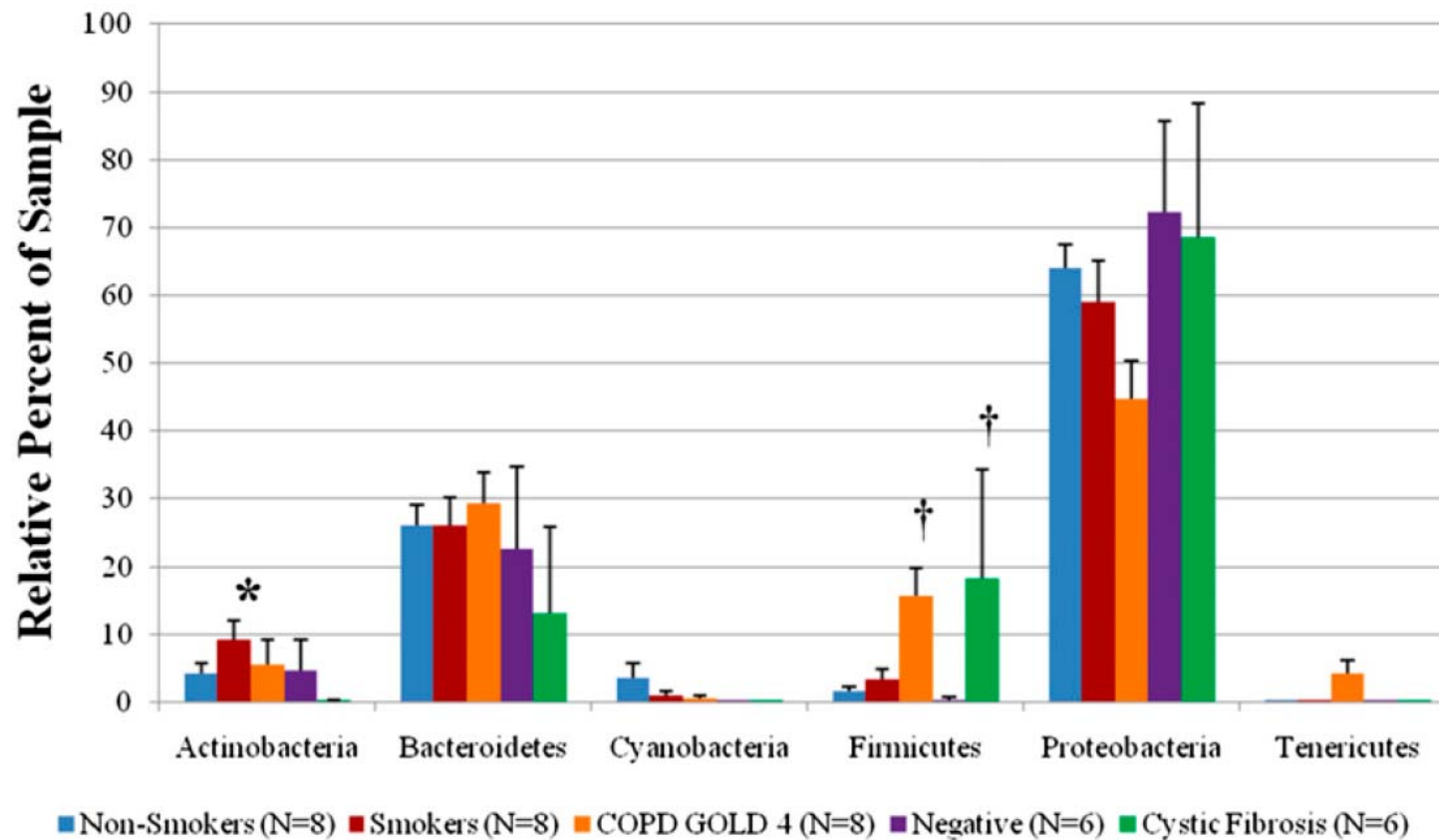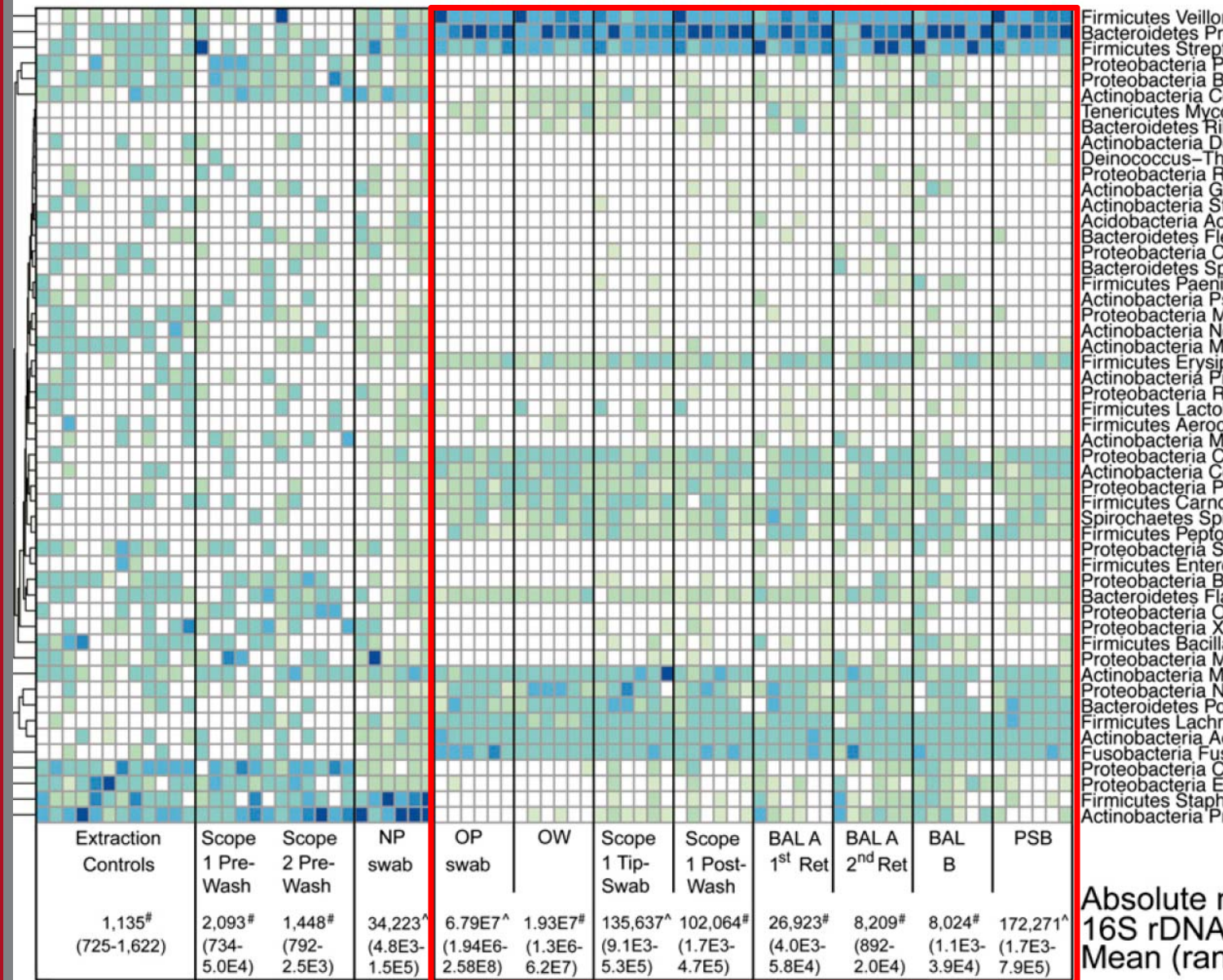# Lungs have fewer bacteria than oropharynx
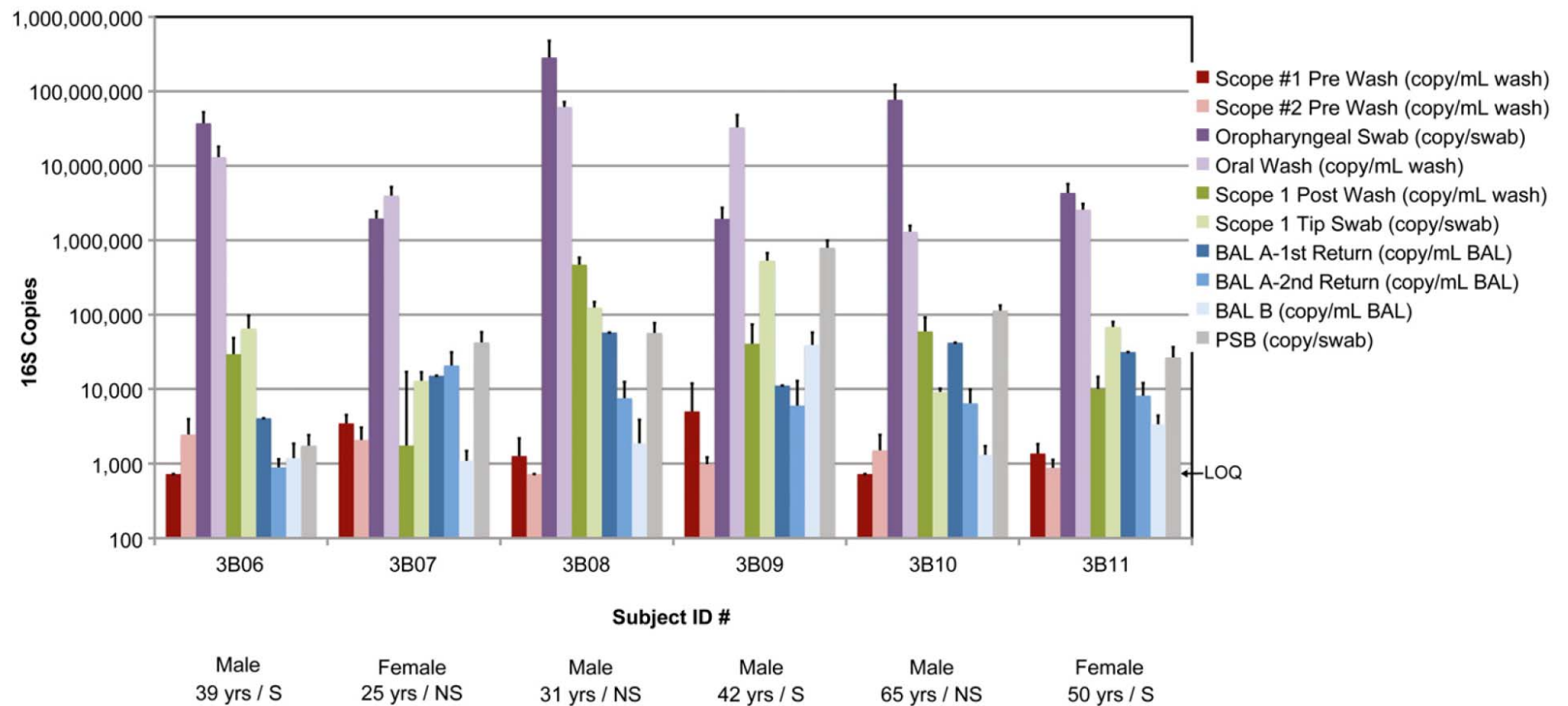
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Which microbiome? Examples

- Environment
  - Target for intervention in asthma?

- Lung
  - Biomarker for COPD morbidity?

- Gut
- Nasal
- Oral

The environmental microbiome and asthma

Amish and Hutterite are farmers

Amish

Hutterite

Dust from Amish homes protects against asthma

asthma model
(ovalbumin)

+ House dust extract (Amish) = ↓ bronchial hyper-reactivity

+ No dust = control

+ House dust extract (Hutterite) = ↑ bronchial hyper-reactivity

"What is in that magic dust?"

Endotoxin Levels in Airborne Dust

Amish | Hutterite

Corynebacteriaceae (0.026 vs. 0.067)
Micrococcaceae (0.045 vs. 0.04)
Prevotellaceae (0.001 vs. 0.022)
Bacillaceae (0.041 vs. 0.021)
Staphylococcaceae (0.037 vs. 0.08)
Lactobacillaceae (0.003 vs. 0.02)
Streptococcaceae (0.022 vs. 0.065)
Clostridiaceae (0.007 vs. 0.046)

Lachnospiraceae (0.005 vs. 0.026)
Ruminococcaceae (0.014 vs. 0.047)
Bartonellaceae (0.347 vs. 0)
Rhodobacteraceae (0.025 vs. 0.016)
Sphingomonadaceae (0.033 vs. 0.042)
Enterobacteriaceae (0.029 vs. 0.021)
Moraxellaceae (0.032 vs. 0.026)
Other (0.333 vs. 0.463)

School microbiome pilot study

Study design: Pilot RCT

- School: Integrated pest management (IPM)
- Classroom: real vs sham HEPA air cleaners

Population: 25 children with persistent asthma

Primary outcome: Asthma symptom days over prior 14 days

Primary exposures: Longitudinal vacuumed dust samples from classrooms and homes → shotgun sequencing

Aims

1. Is the school microbial environment different from the home microbial environment?
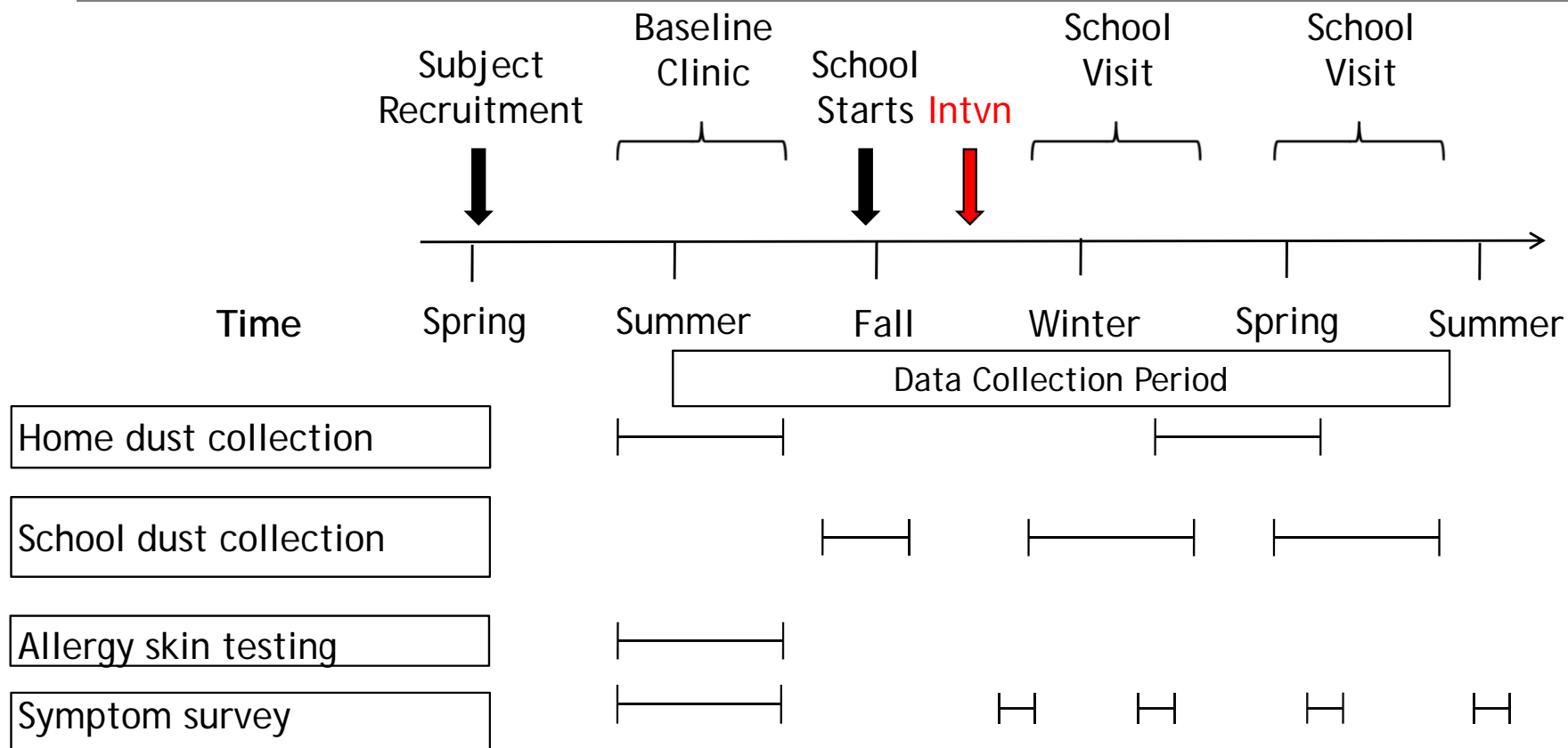   - Microbial identity
   - Microbial function (Lipid A biosynthesis)

2. Is the school microbial environment independently associated with asthma symptoms?

3. Is the school microbial environment modifiable?

Study schema

School microbiome pilot study

Sample processing:
- Microbial DNA extraction using a modified cetyltrimethylammonium bromide–polyethylene glycol (CTAB) extraction protocol
- Shotgun metagenomic sequencing on Illumina HiSeq 2500 platform, 2x101 bp paired end reads targeting ~2Gb of sequence per sample

Bioinformatics/Statistical approach:
- kneadData to remove contaminating human reads
- MetaPhlAn2 for taxonomic profiling, HUMAnN2 for functional profiling
- 5% prevalence filter to exclude sequencing artifact and spurious taxa
- *phyloseq* to characterize microbial community structure and to calculate diversity measures
- *vegan* for permutational analysis of variance (PERMANOVA)
- *gamm4* for generalized additive mixed effects models to evaluate the association between classroom microbial richness and asthma symptom days
  - Microbial richness measured by Inverse Simpson index
  - Season modelled with penalized spline term on days since school start
  - Adjusting for gender, age, race, home microbial richness

School and home microbiome is different
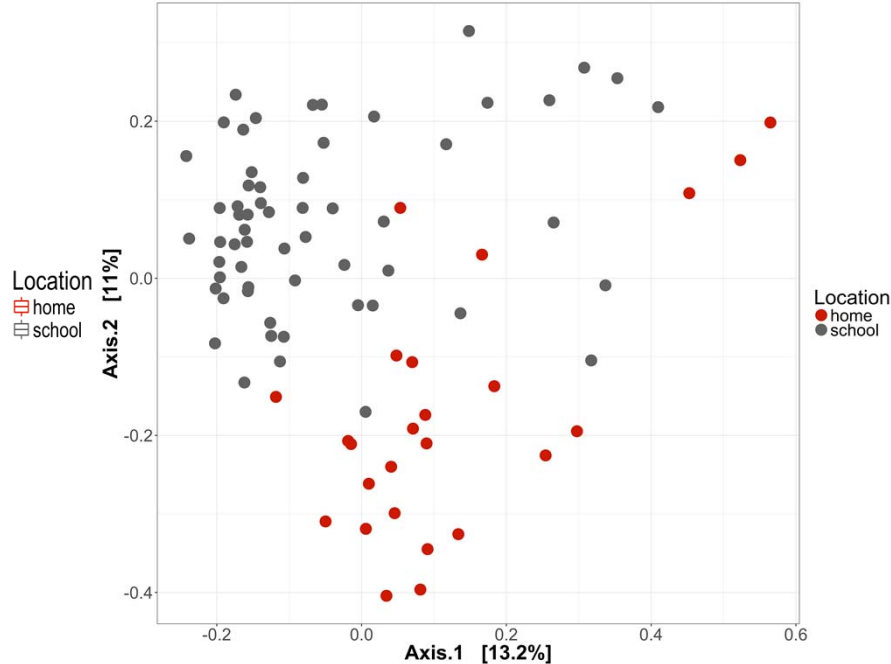
1a. Classrooms have higher α diversity
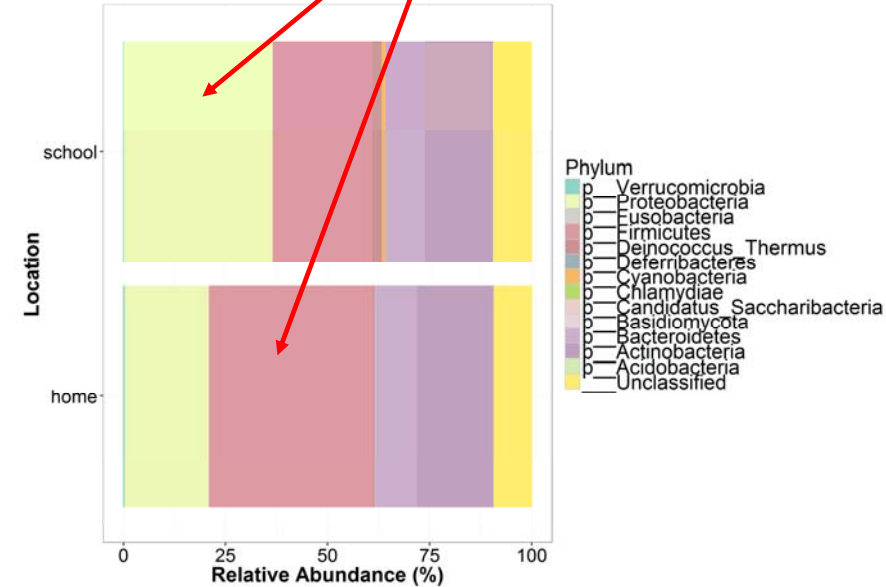
β = 6.79 [0.63 - 12.95], p = 0.03
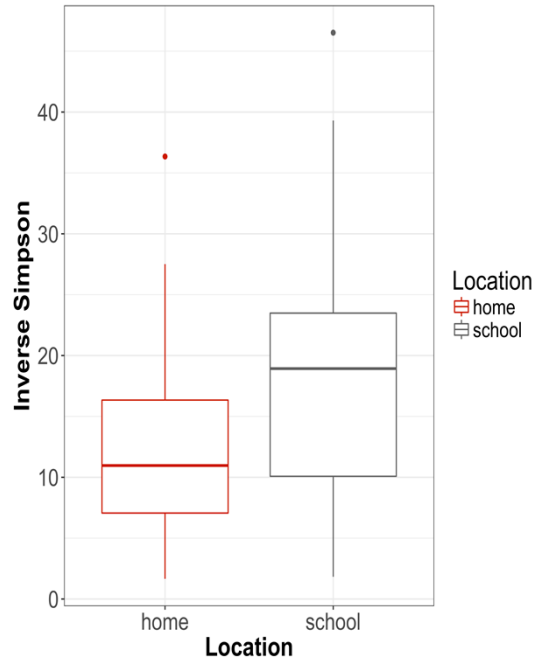
1b. PCoA of Bray-Curtis disimilarity

PERMANOVA R^2 = 0.08, p = 0.001

1c. Schools have more Proteobacteria, homes have more Firmicutes

Different bacterial species contributing to Lipid A biosynthesis in homes vs schools

	Home	Classroom	p-value
n	26	78	
Citrobacter freundii (mean (sd))	0.95 (4.87)	0.20 (1.05)	0.202
Enterobacter cloacae (mean (sd))	3.30 (6.84)	0.44 (2.39)	0.002
Escherichia coli (mean (sd))	1.65 (6.92)	0.00 (0.00)	0.036
Haemophilus parahaemolyticus (mean (sd))	0.00 (0.00)	0.08 (0.70)	0.566
Haemophilus parainfluenzae (mean (sd))	1.37 (4.15)	2.83 (18.05)	0.684
Klebsiella oxytoca (mean (sd))	4.95 (20.85)	0.53 (1.81)	0.064
Klebsiella pneumoniae (mean (sd))	0.31 (1.09)	0.96 (6.66)	0.622
Pantoea agglomerans (mean (sd))	0.16 (0.81)	0.23 (1.07)	0.764
Pantoea ananatis (mean (sd))	0.00 (0.00)	0.02 (0.16)	0.566
Pantoea dispersa (mean (sd))	0.14 (0.72)	0.29 (2.10)	0.721
Raoultella ornithinolytica (mean (sd))	0.00 (0.00)	0.07 (0.45)	0.415
Serratia marcescens (mean (sd))	0.00 (0.00)	7.01 (19.56)	0.072

School microbial diversity associated with asthma symptoms

	Odds Ratio [95% CI]	p-value
Male vs. female	0.92 [0.29 - 2.98]	0.89
Age in years	1.33 [0.86 - 2.04]	0.20
Hispanic vs. black race	1.58 [0.42 - 5.9]	0.49
Other vs. black race	0.77 [0.15 - 4.02]	0.75
Classroom microbial diversity [b]	1.07 [1.00 - 1.14]	0.05 [c]
Home microbial diversity [b]	1.00 [1.00 - 1.00]	0.91
Season [d]	0.74 [0.38 - 1.47]	0.39

Generalized additive mixed effects model with asthma symptom days as outcome, penalized spline term on time to adjust for season
[c] p = 0.0469

Integrated pest management intervention associated with classroom microbiome

	R^2	p-value
IPM	0.03499	0.002
HEPA filtration	0.01781	0.27
Classroom ID	0.38837	0.001

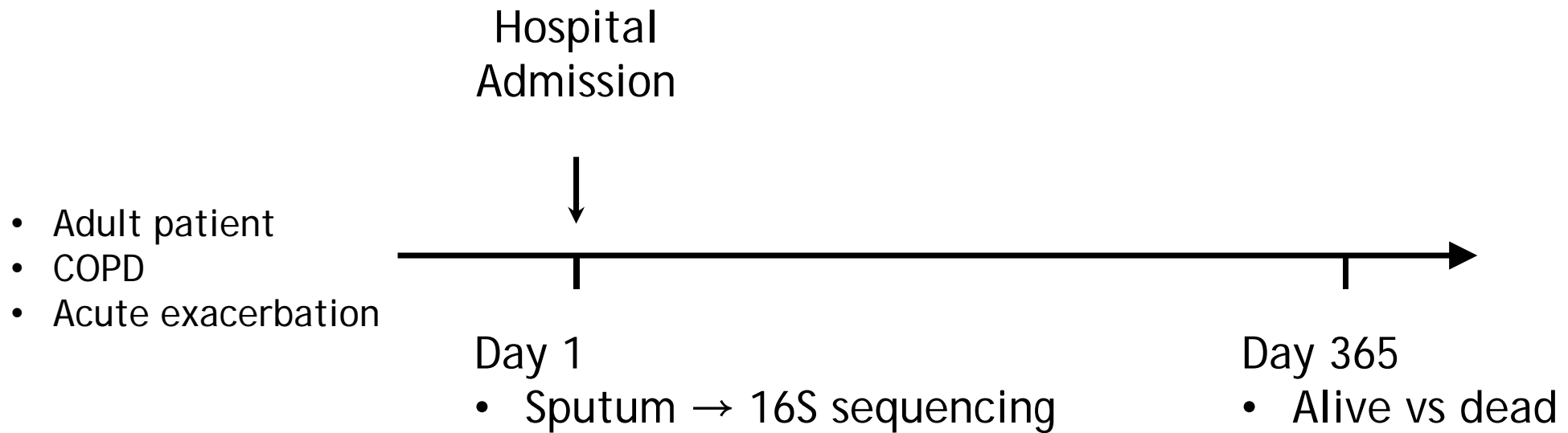PERMANOVA on Bray-Curtis dissimilarity of sequenced vacuum dust samples

Which microbiome? Examples

- Environment
  - Target for intervention in asthma?
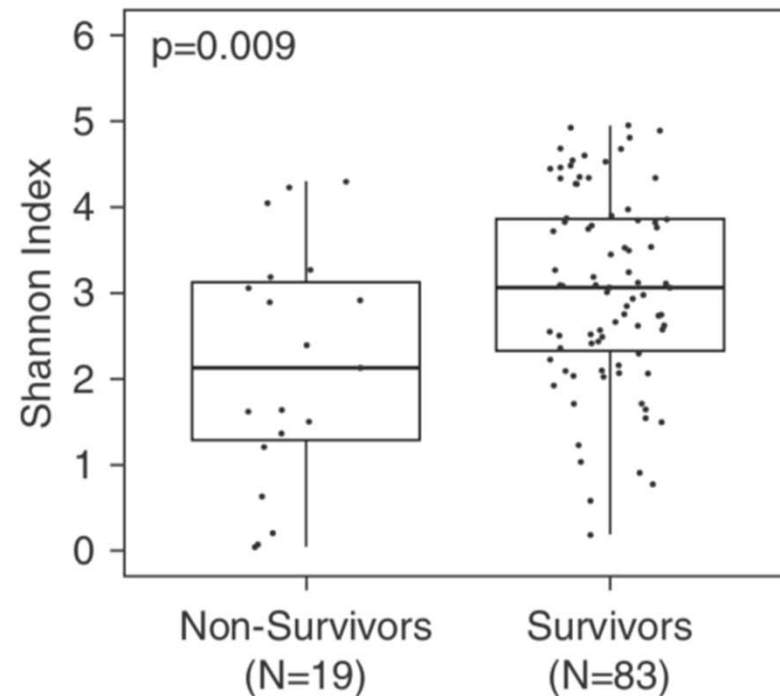- Lung
  - Biomarker for COPD morbidity?

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- Nasal
- Oral
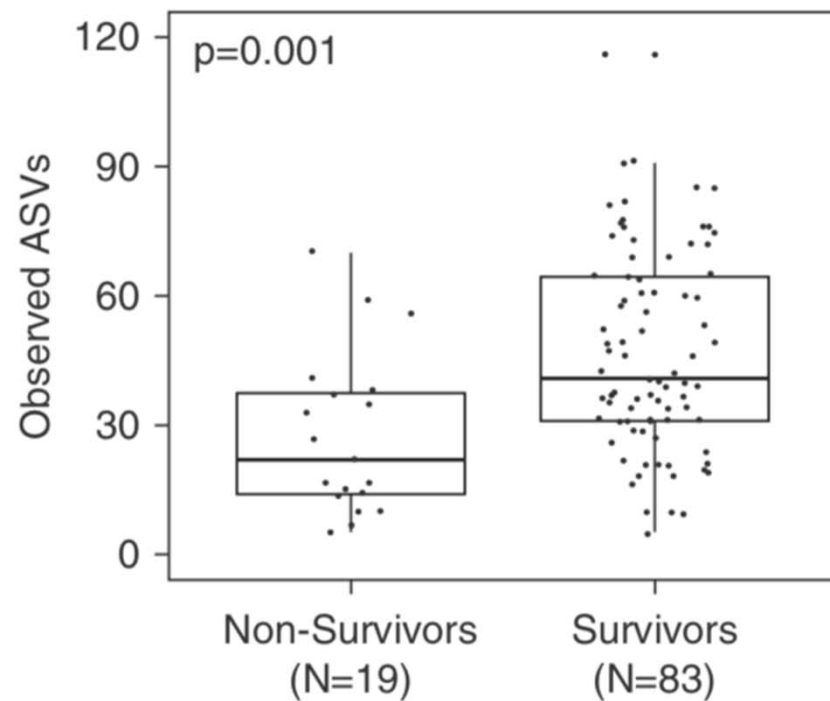
Sputum microbiome as a biomarker of mortality in acute COPD exacerbation

Hospital Admission

- Adult patient
- COPD
- Acute exacerbation

Day 1
- Sputum → 16S sequencing

Day 365
- Alive vs dead

Survivors had higher α diversity in sputum

Lower α diversity associated with higher risk of death

Table 2. Adjusted Hazard Ratios of Different α-Diversity Metrics for 1-Year Mortality after Admission due to Acute Exacerbation of Chronic Obstructive Pulmonary Disease

α-Diversity Metric	Coefficient	HR*	95% CI	*P* Value
Observed ASVs	−0.045	0.96	0.93–0.98	0.002
Shannon index	−0.625	0.53	0.35–0.81	0.003
Faith's Phylogenetic Diversity	−0.475	0.62	0.47–0.83	0.001

Definition of abbreviations: ASV = amplicon sequence variant; CI = confidence interval; HR = hazard ratio.
*HRs obtained after adjustments for age, sex, smoking status, long-term oxygen therapy, history of acute exacerbations in the previous year, use of antibiotics during admission, and comorbidity burden (according to the Seattle Index of Comorbidity [30]).

Differences in abundance of specific taxa in survivors vs non-survivors
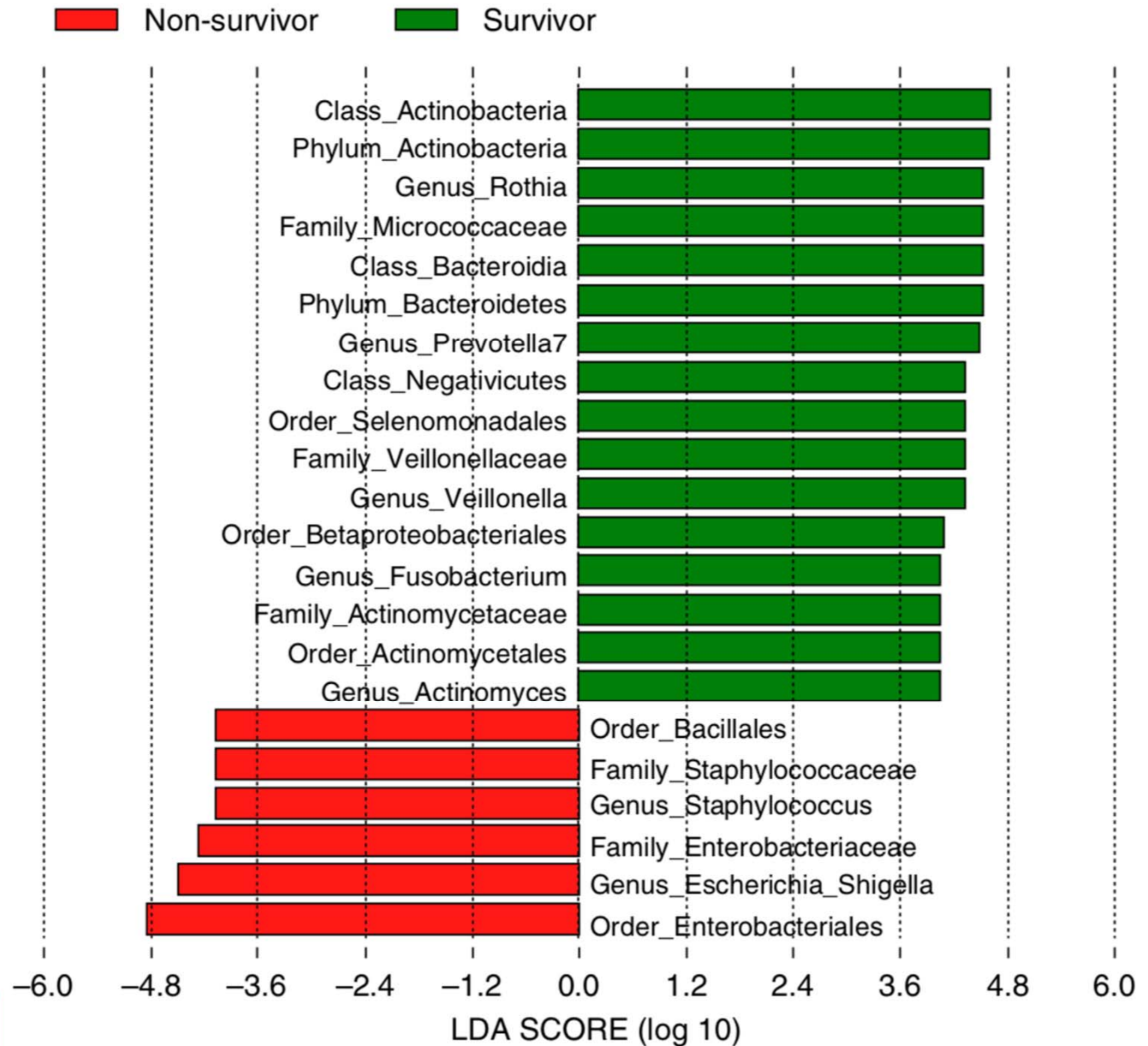
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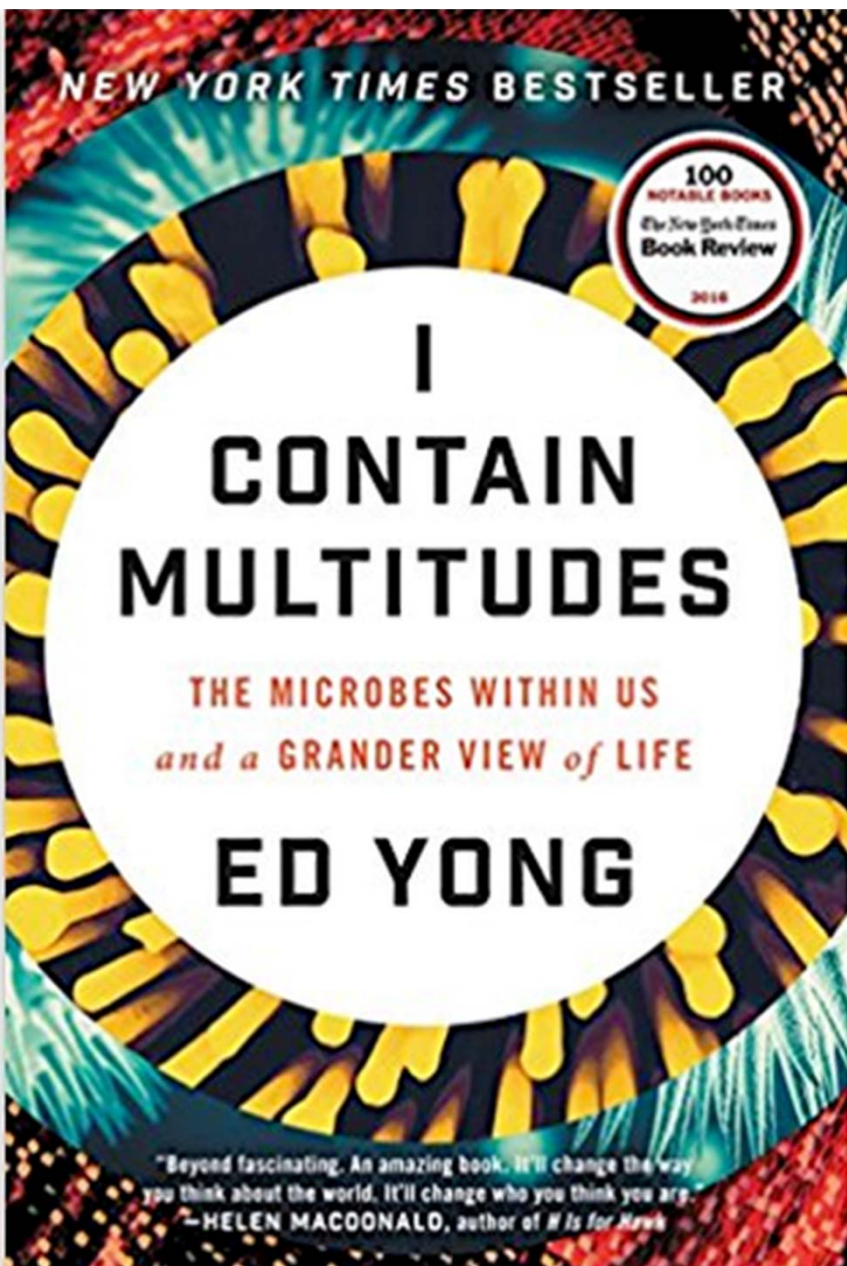
Suggested Reading

Suggested Reading

Morgan XC, Huttenhower C. Human microbiome analysis. PLoS Comp Bio 2012. PMID: 23300406

Knight R, et al. Best practices for analyzing microbiomes. Nat Rev Microbiology 2018. PMID: 29795328.

Kim D, Hofstaedter CE, Zhao C et al. Optimizing methods and dodging pitfalls in Microbiome Research. Microbiome 2017. PMID: 28476139

Faner R, Sibila O, Agusti A, et al. The microbiome in respiratory medicine: current challenges and future perspectives. Eur Respir J 2017. PMID: 28404649.

Carney et al. Methods in Lung Microbiome Research. AJRCMB 2019. PMID: 31661299.