CULTURE INDEPENDENT ANALYSIS OF THE MILK MICROBIOME

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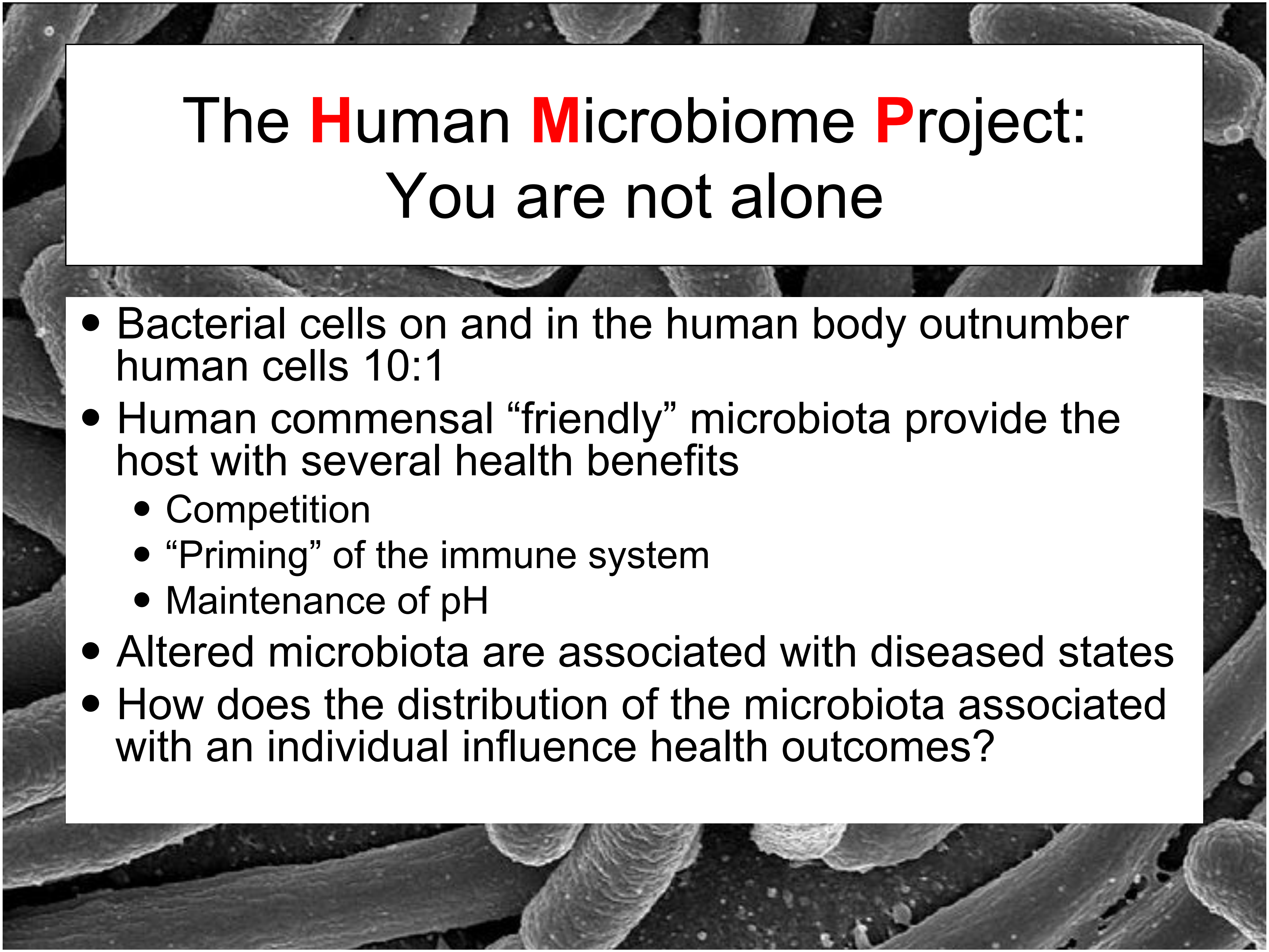
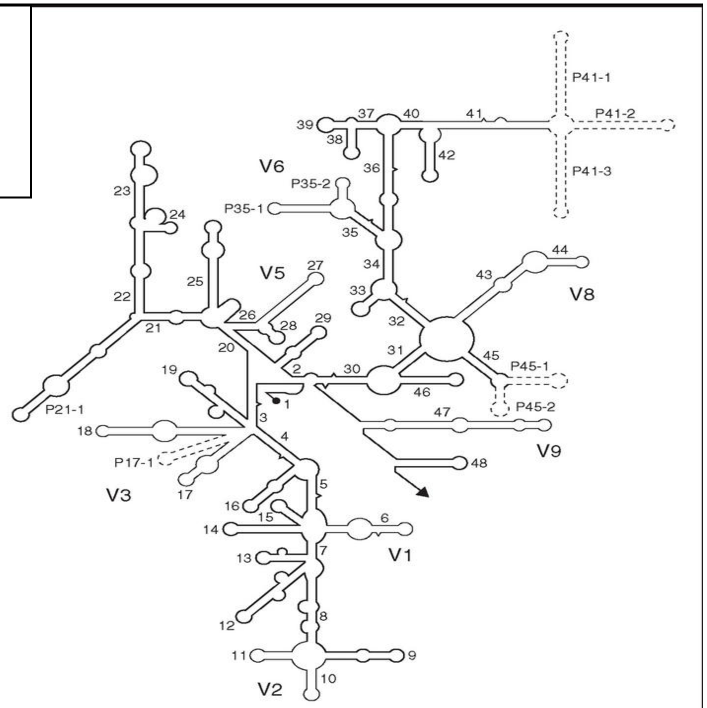


The **H**uman **M**icrobiome **P**roject: You are not alone

- Bacterial cells on and in the human body outnumber human cells 10:1
- Human commensal “friendly” microbiota provide the host with several health benefits
  - Competition
  - “Priming” of the immune system
  - Maintenance of pH
- Altered microbiota are associated with diseased states
- How does the distribution of the microbiota associated with an individual influence health outcomes?

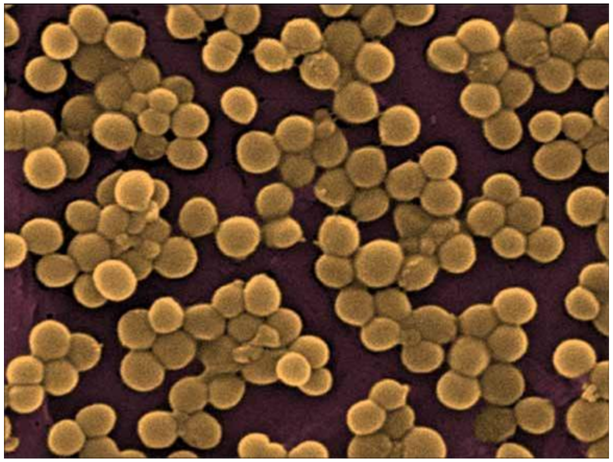
16S Ribosomal RNA



- Unique to bacteria: humans have 18S → differentiation between host and bacterial gene
- Conserved regions allow for use of universal primers
- Hypervariable regions allow for differentiation between bacterial types
- Comparing sequences to databases allows for determination of bacterial classification
- Snapshot of community composition

Bacterial Categorization

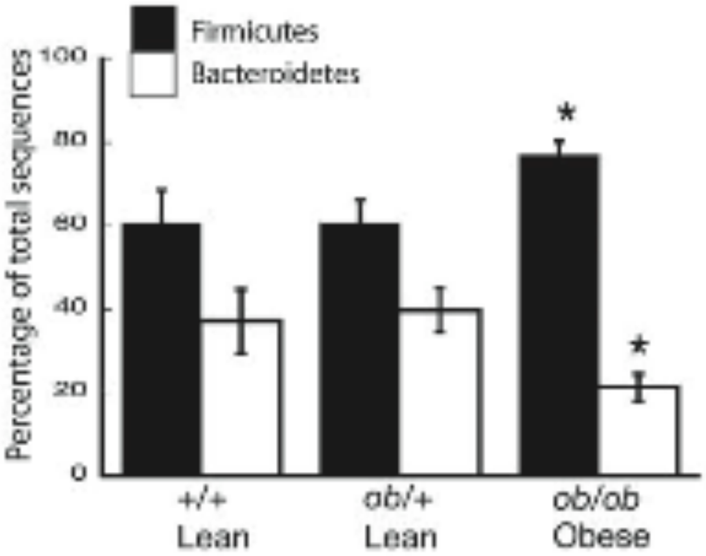
- Phylotypes
- Genera
- Species (97% sequence identity)
- Operational Taxonomic Unit (OTU)



Studies in ob/ob mice: Obesity is related to an altered microbiota in the distal colon:

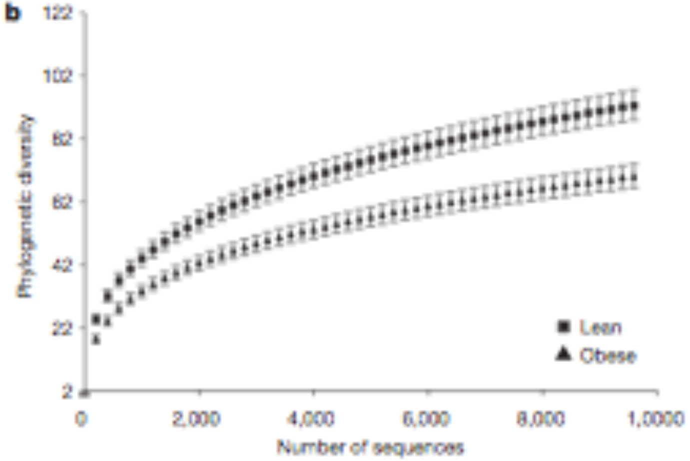
- ob/ob mice display a 50% reduction in the abundance of Bacteroidetes and a corresponding increase in proportion of Firmicutes

(Ley et al. 2005)

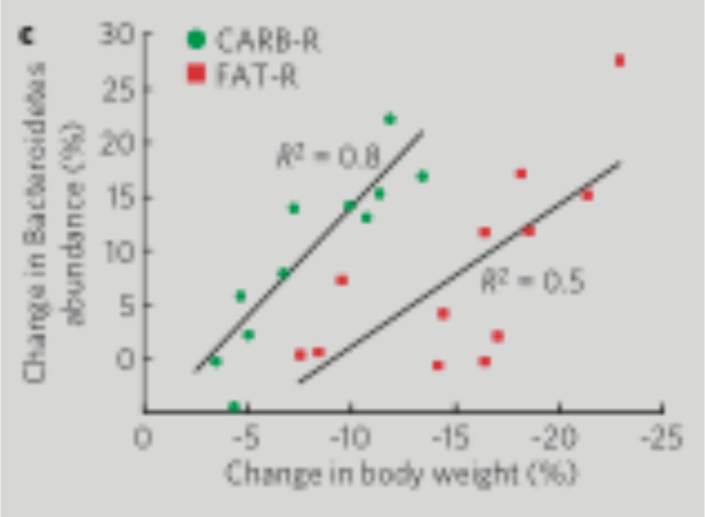


A human gut microbiome associated with obesity

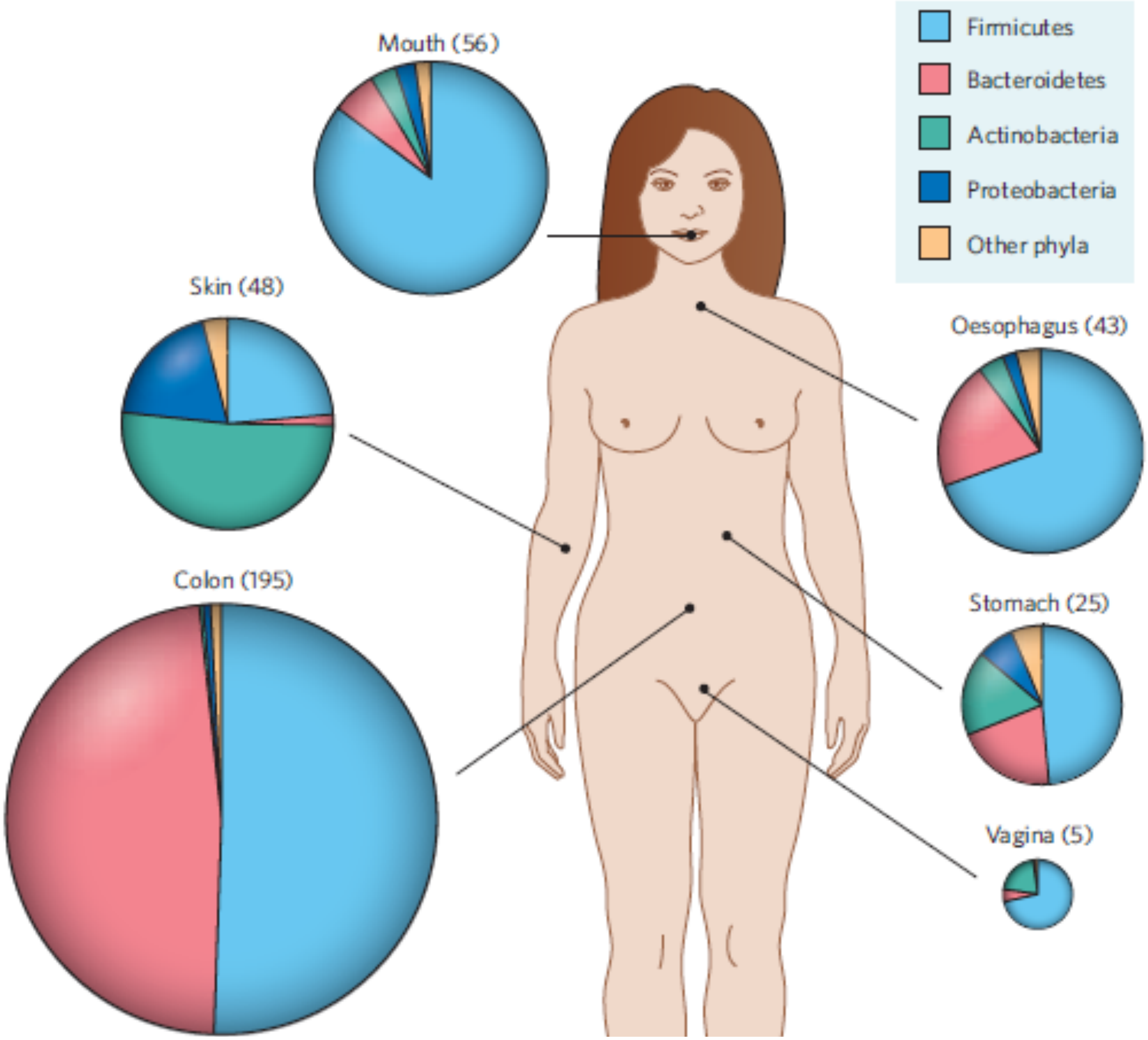
- In a study analyzing the fecal microbiota of 154 human twins (mono and dizygotic), obesity was related to decreased bacterial diversity and changes in community composition at the phylum level (Turnbaugh et al. 2009)



- In a study where 12 obese individuals were assigned to fat or carbohydrate restricted low calorie diets:
    - The relative abundance of Bacteroidetes increased and the abundance of Firmicutes decreased.
    - Increase in Bacteroidetes correlated with loss of weight (Ley et al. 2006)



Got milk?



Dethlefsen et al (2007) Nature

Gut microbiota of breastfed compared with formula-fed infants

More	Equal	Less
Staphylococcus *Lactobacillus rhamnosus**	*Bifidobacterium* *Lactobacillus*	*Clostridium (C. difficile)* *Bacteroides* *Enterobacteriaceae* *Enterococcus*

Exception within the genus Lactobacillus

Vael and Desager, 2009, Curr Opin Pediatr 21:794-800

Benefits of Breastfeeding: Links to Bacteria

- Reduced respiratory infection
- Reduced gastrointestinal disease
- 20% reduction in risk of childhood obesity
  - Koletzko et al. (2009) Adv Exp Med Biol 646:15

Cultivation dependent determination

- Greater than 90% of bacteria have <u>never</u> been cultured
- Growth on limited media
  - Focus on pathogens
- 30-35% of milk samples from cows or women with mastitis fail to grow any bacteria
  - Might not be bacterial in nature
- Anaerobic bacteria rarely are routinely cultured

These methods are <u>not</u> unbiased!

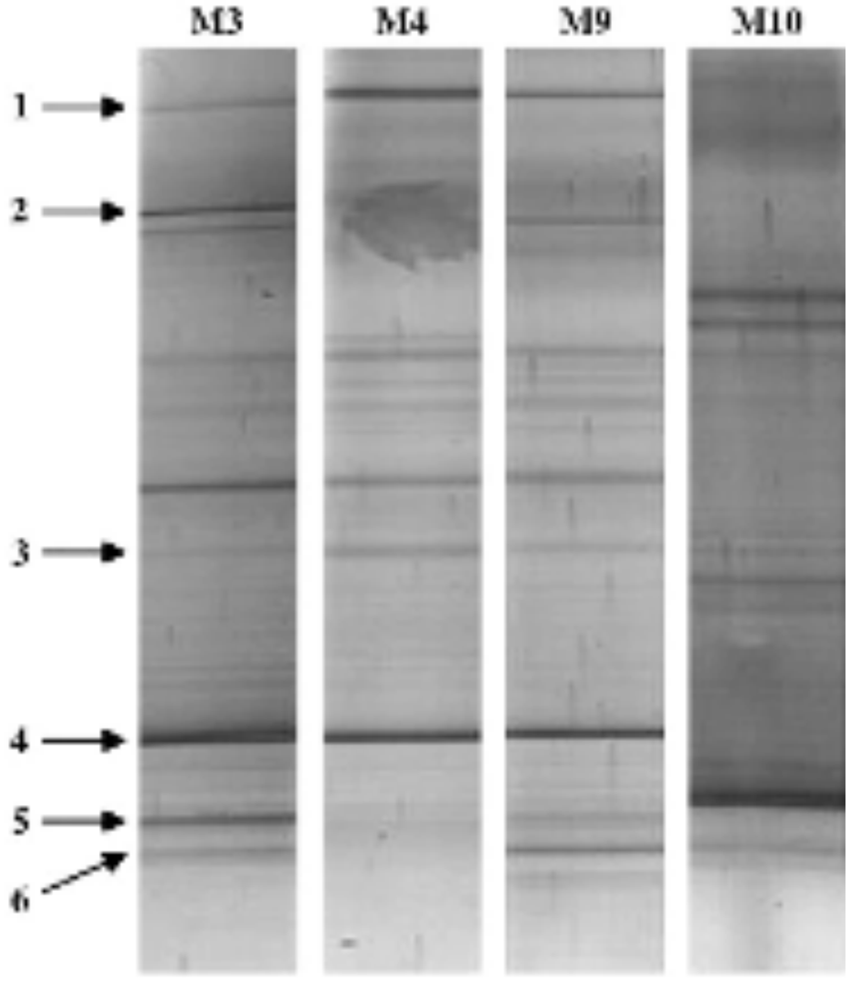
Cultivation independent assessments: healthy human milk is not sterile

- Milk from five mothers who delivered vaginally and five who delivered by elective cesarean

- Amplification of the V6-V8 region of the 16S rRNA gene

- Milk samples from healthy mothers contains 2-5 dominant types of bacteria (lactic acid bacteria, Streptococci, Staphylococci, E. coli, Pseudomonas)

- The patterns of bacterial phylotypes appeared to be host specific



Martín et al. (2007) Res Microbiol 158:31

Cultivation independent assessments: healthy human milk is not sterile

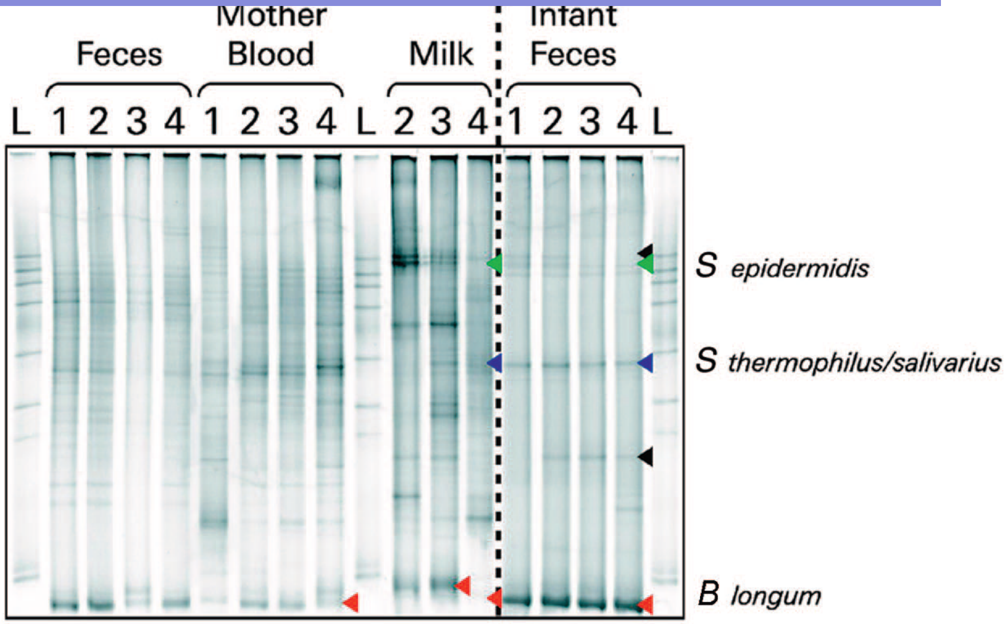
- Milk from healthy mothers

- 2-3 mL foremilk discarded, breast cleaned with antiseptic soap, rinsed distilled water and dried sterile gauze before aseptic collection with electric pump

- Amplification of the V6-V8 region of the 16S rRNA gene

- Contained Lactobacillus, Streptococcus, Enterococcus, Peptostreptococcus, Staphylococcus, Corynebacterium and occasionally Escherichia spp.

- Total concentration of 10^3 colony-forming units per mL



Perez et al. (2007) Pediatrics 119:e724

Suggested link between maternal gut and breast

Enterohumoral transfer through maternal blood mononuclear cells to milk cells

Microbiome assessment

- Samples collected from 16 women at three time-points
- Culture dependent analysis following National Mastitis Council procedures
- Culture independent bacterial analysis of the samples was performed
  - DNA extraction
  - PCR amplification of 16S rRNA
  - Pyrosequencing of amplicons
  - Computational analysis

Cultivation dependent results

- The culture based results identified only a handful of bacterial species- and **roughly 20% of samples were void of bacterial growth altogether.**
- Top three phylotypes were *Streptococci*, *Staphylococci* and *Corynebacterium*

Pyrosequencing data analysis

454 sequencing produced 448,051 reads

Sequences were assigned to samples by barcodes. Processing removed low quality reads, those with ambiguous bases or large homopolymers, and reads that did not contain a correct barcode or primer. This left 435,036 reads (97% of the original data set)

Sequences were aligned to the V2 region of the 16S gene using the SILVA database, those that did not align were removed. This left 64,391 unique sequences representing 301,867 total reads (67% of the original data set).

The Chimera Slayer program was then used to remove potential chimeras from the data set

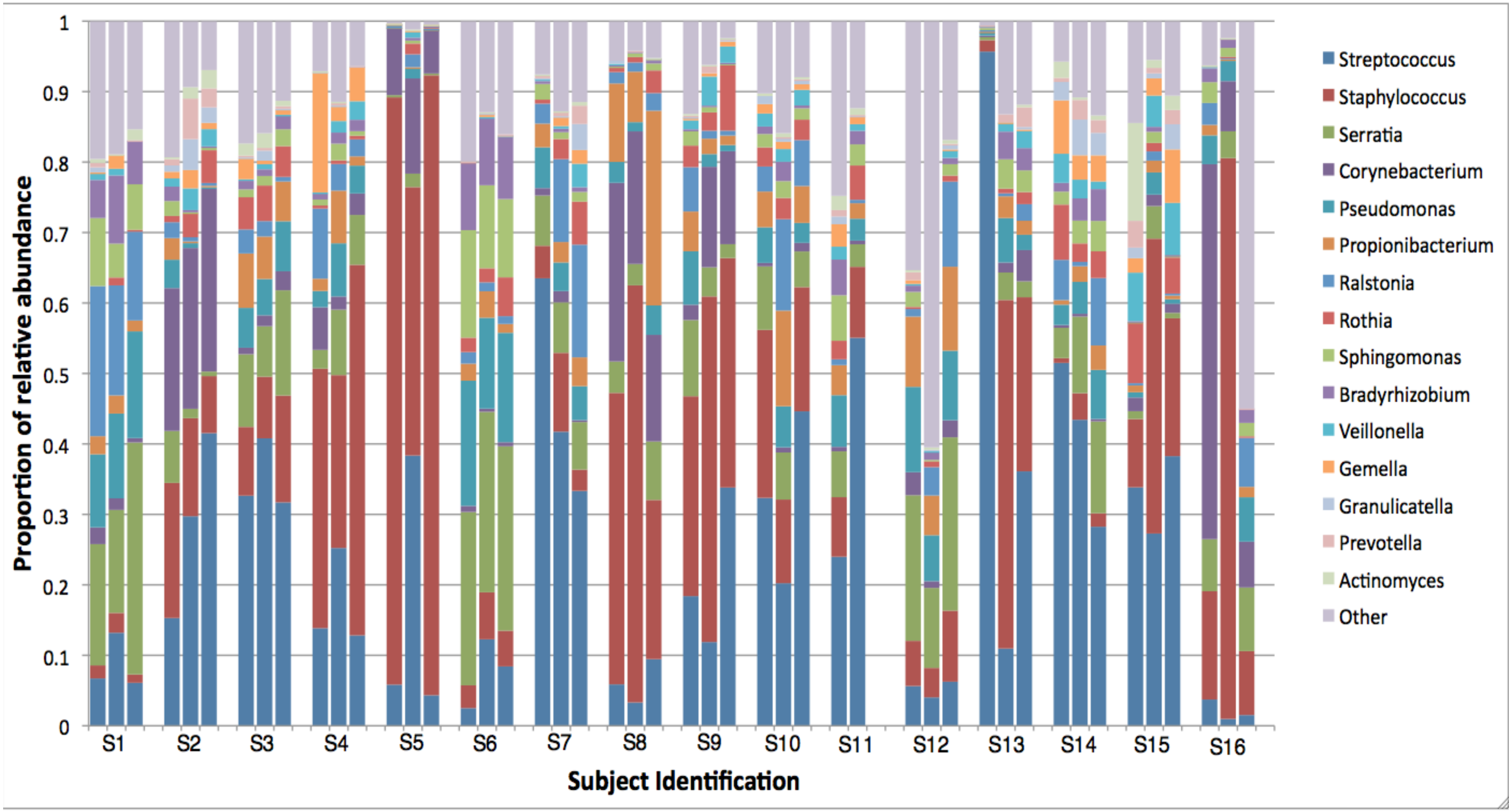
The final result of quality control produced 230,000 sequences (60% of the original data set) for use in downstream analysis.

Pyrosequencing summary statistics

- 454 FLX Sequencing produced over 230,000 reads
- Average of ~4,000 per sample
- Dominant genera: *Streptococcus*, *Staphylococcus, Serratia* and *Corynebacterium*
- Several genera observed in every milk sample without exception
- *Bifidobacteria* or *Lactobacillus*?

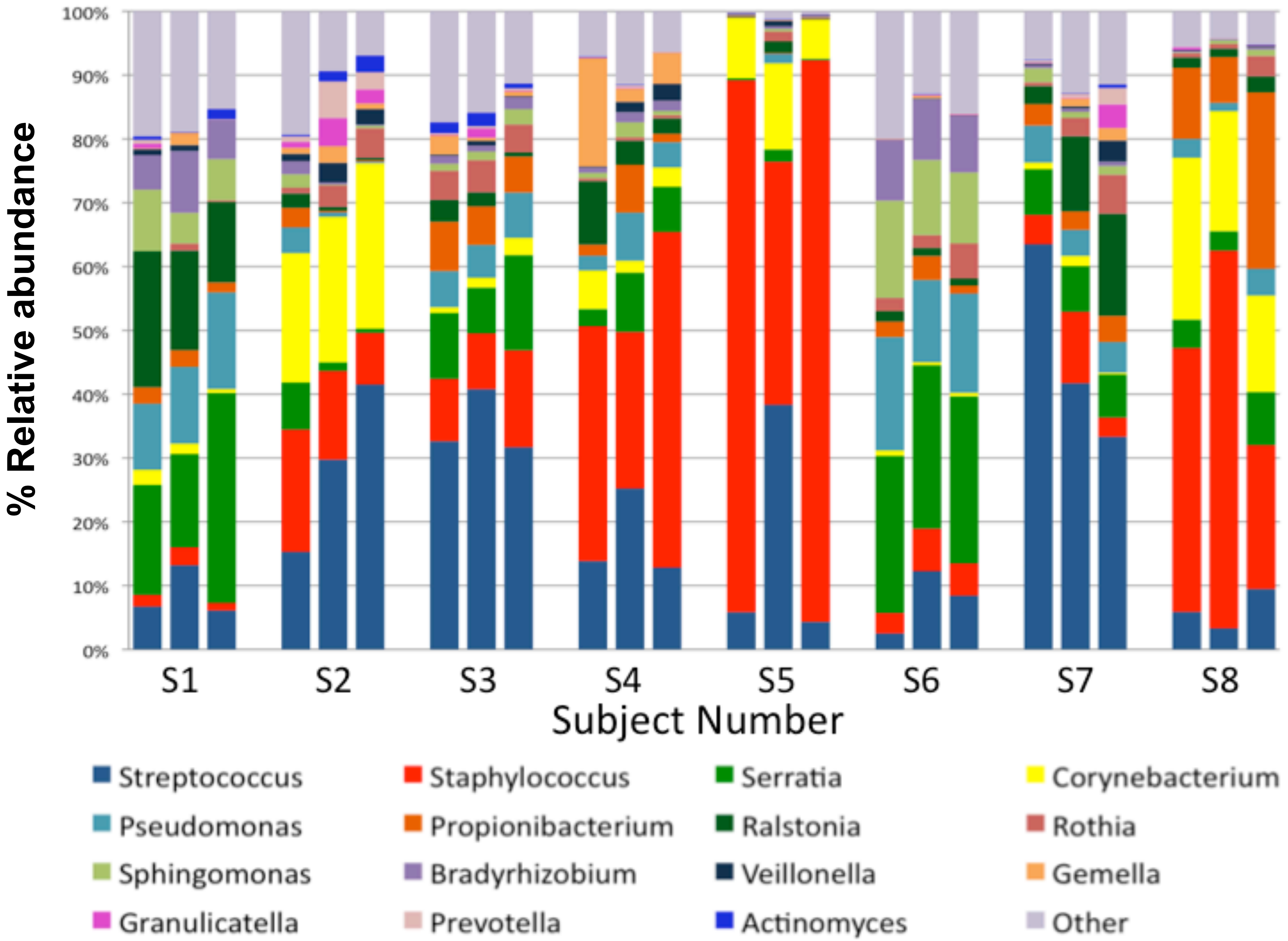
Hunt et al (2011) PLoS ONE 6:e21313

Relative abundance of the top 15 bacterial genera in 16 women at three time-points



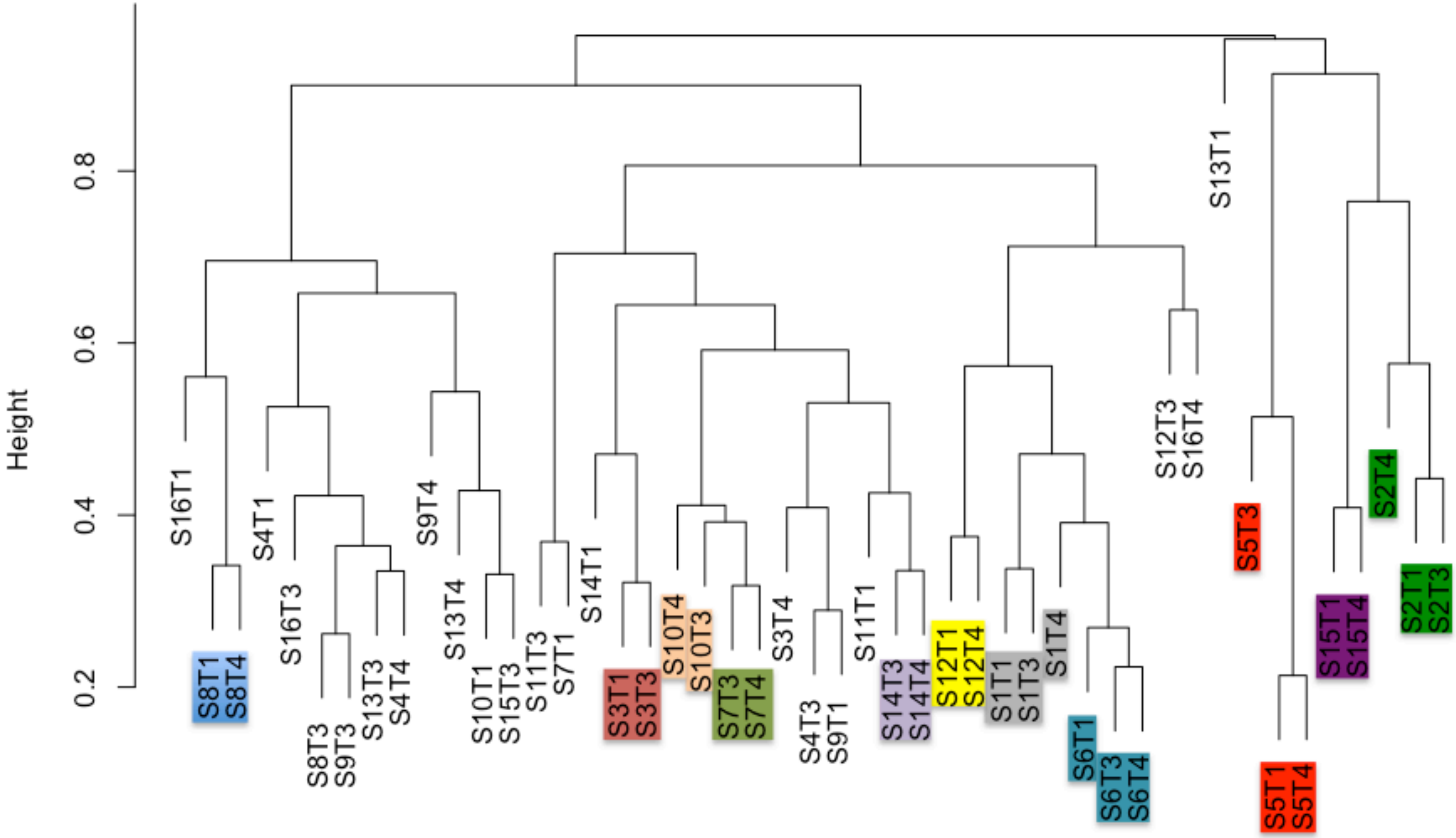
Hunt et al (2011) PLoS ONE 6:e21313

Human milk bacterial communities: 3 time-points, 8 women



Hunt et al (2011) PLoS ONE 6:e21313

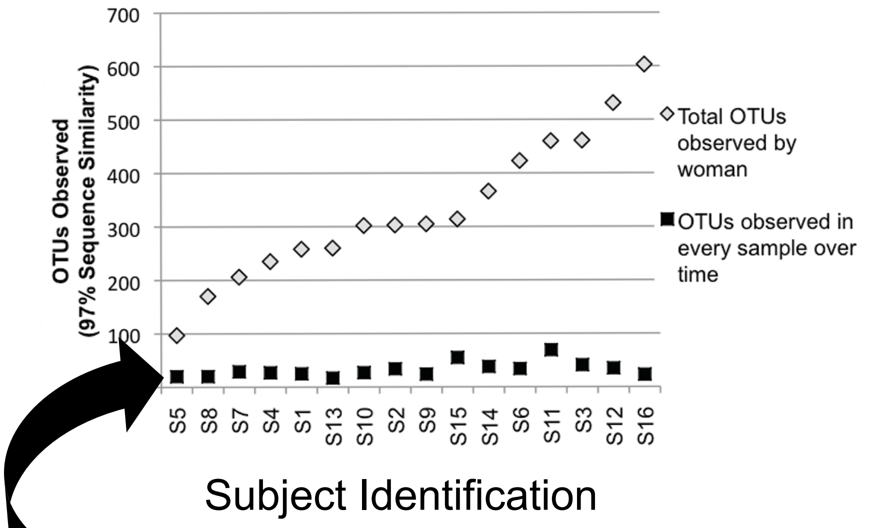
The personalization of milk microbial communities



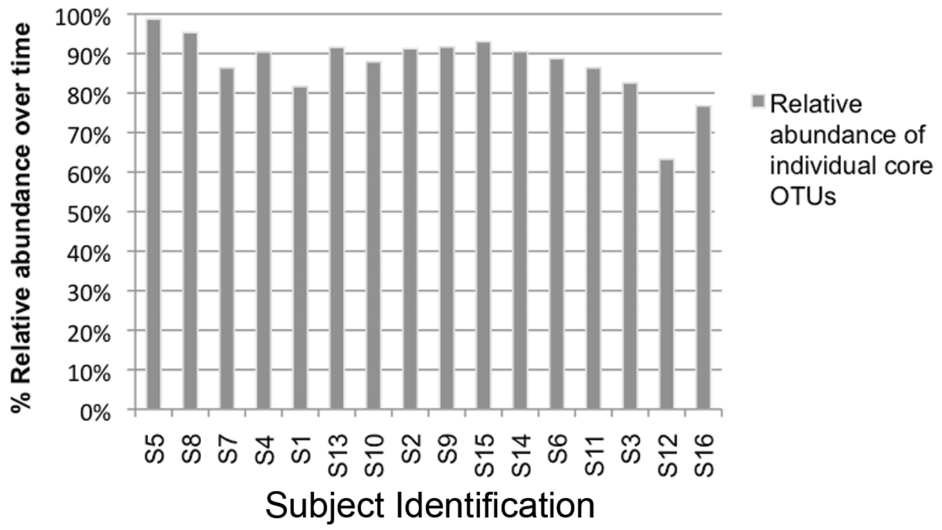
Hunt et al (2011) PLoS ONE 6:e21313

Defining the community:
- **Richness: How many species are present?**
- **Define species on the similarity of the sequences:**
  - **97% sequence similarity = one species**
  - **Operational Taxonomic Units (OTUs)**

What is the richness of OTUs in human milk?
How persistent are these OTUs over time?



What proportion of the community is represented by this "personal core?"



"Personal Core" Milk Microbiome

- **The richness of human milk communities varies among women**
- **~10% of OTUs make up the "personal core"**
- **This core accounts for 85% of the community**

Hunt et al (2011) PLoS ONE 6:e21313

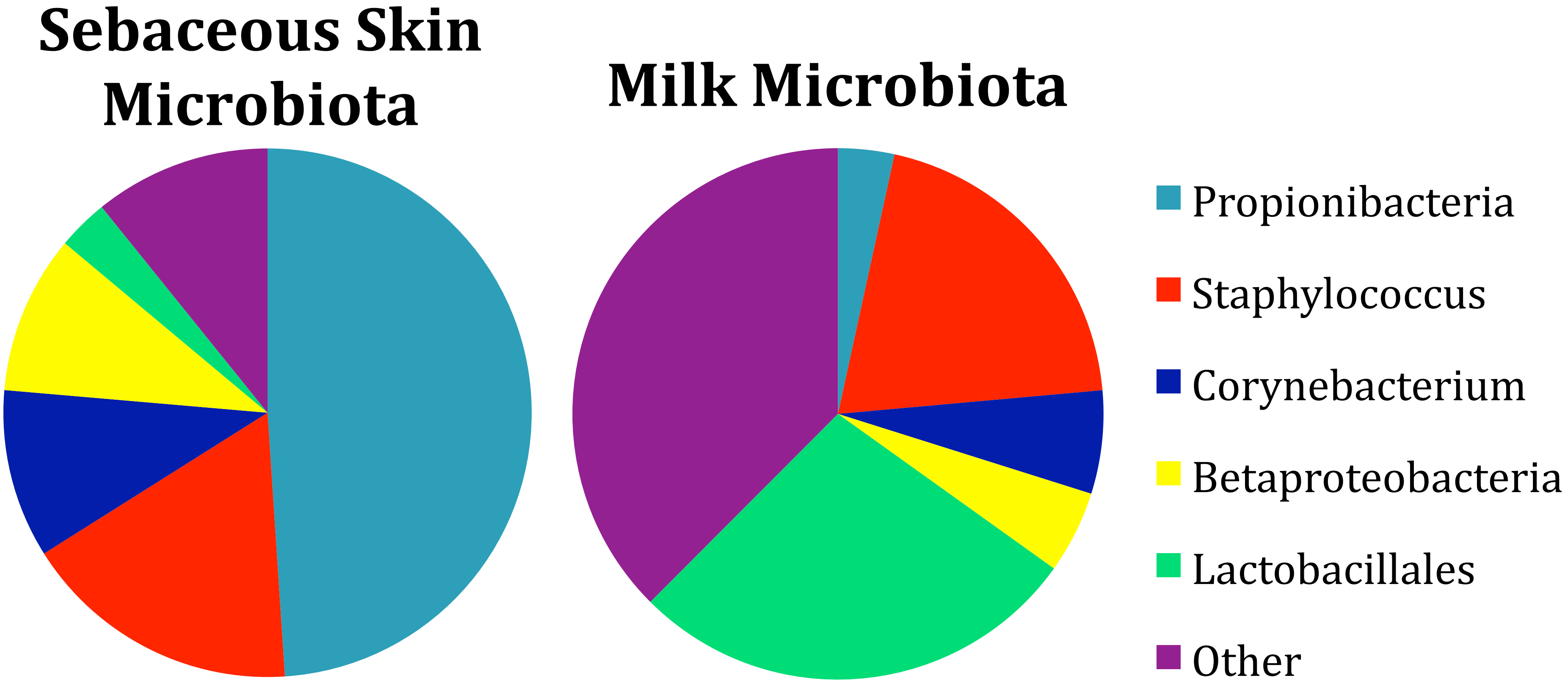
Is there a core milk microbiome among women?

- Are any OTUs present in every sample from every woman?
- What proportion of the community does this core represent?

Core OTU Genera	**Relative abundance (%) in total community**
Staphylococcus	15.8
Streptococcus	8.2
Serratia	7.6
Pseudomonas	4.5
Corynebacterium	3.8
Ralstonia	3.7
Propionibacterium	3.6
Sphingomonas	2.4
Bradyrhizobium	1.9
Sum of all "core" OTUs	**51.5**

Hunt et al (2011) PLoS ONE 6:e21313

Human milk microbiota is not a result of skin contamination



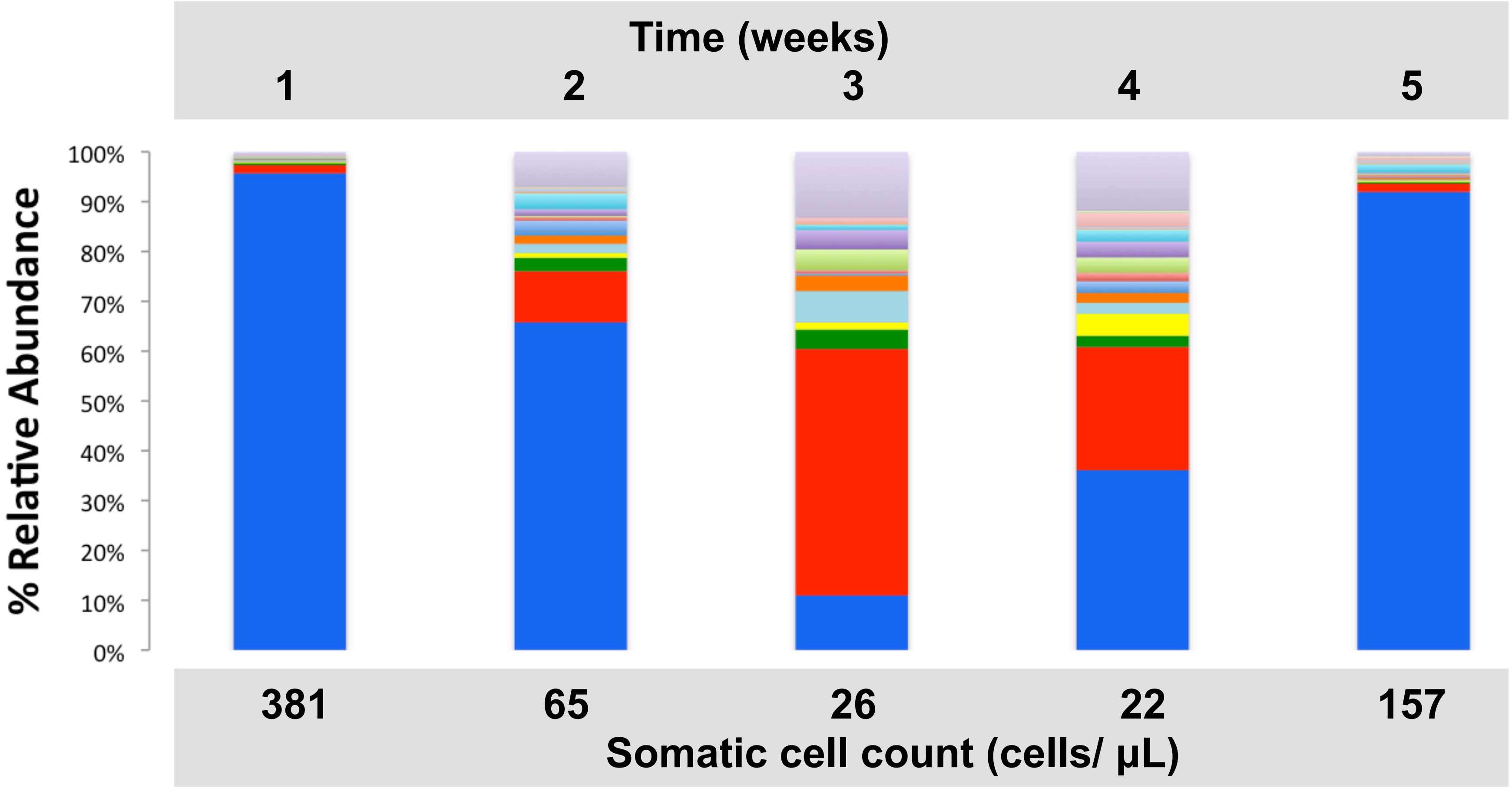
Sebaceous Skin Microbiota

Milk Microbiota

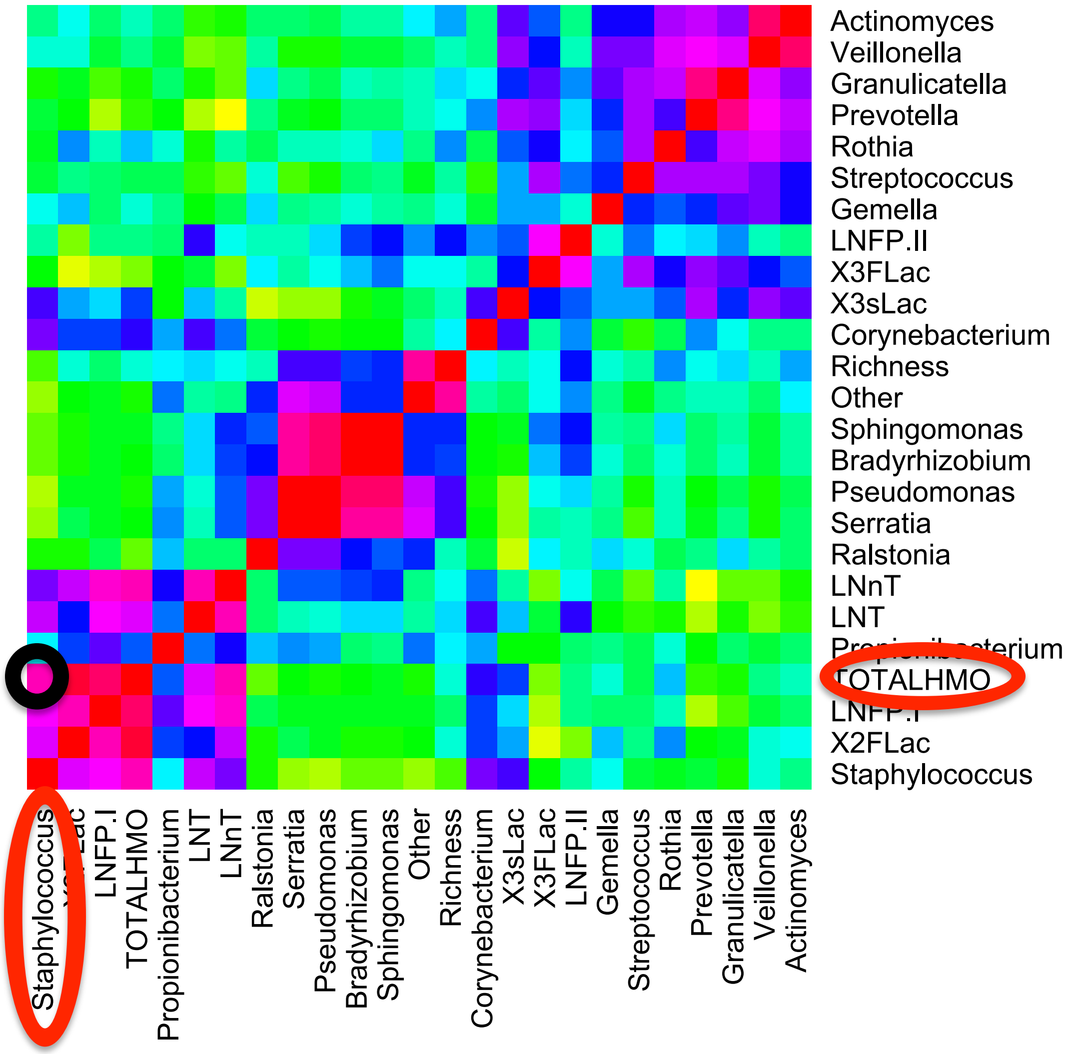
Skin microbiota data from Grice et al. 2009

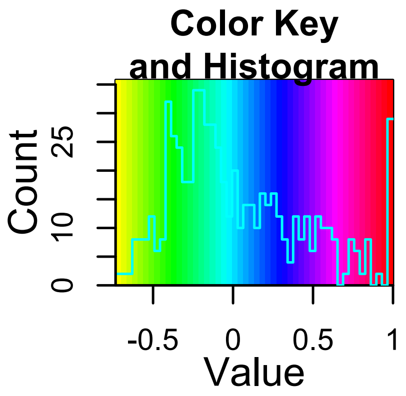
Milk bacterial communities and mastitis

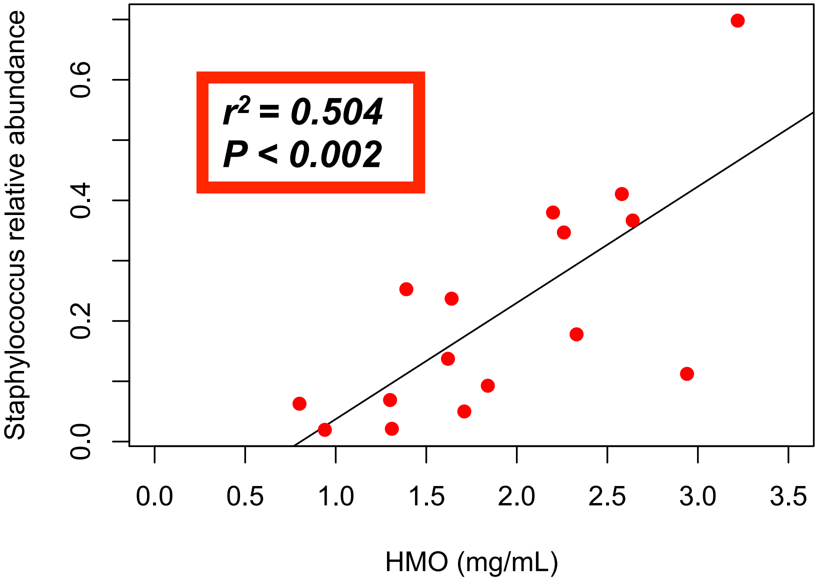
- Samples collected once a week for five weeks
- Subject defined herself as free from mastitis
- Community profile and somatic cell count may tell a different story:



Relationships between milk bacterial communities and milk components?







$r^2 = 0.504$

$P < 0.002$

Effect of HMO on bacterial growth

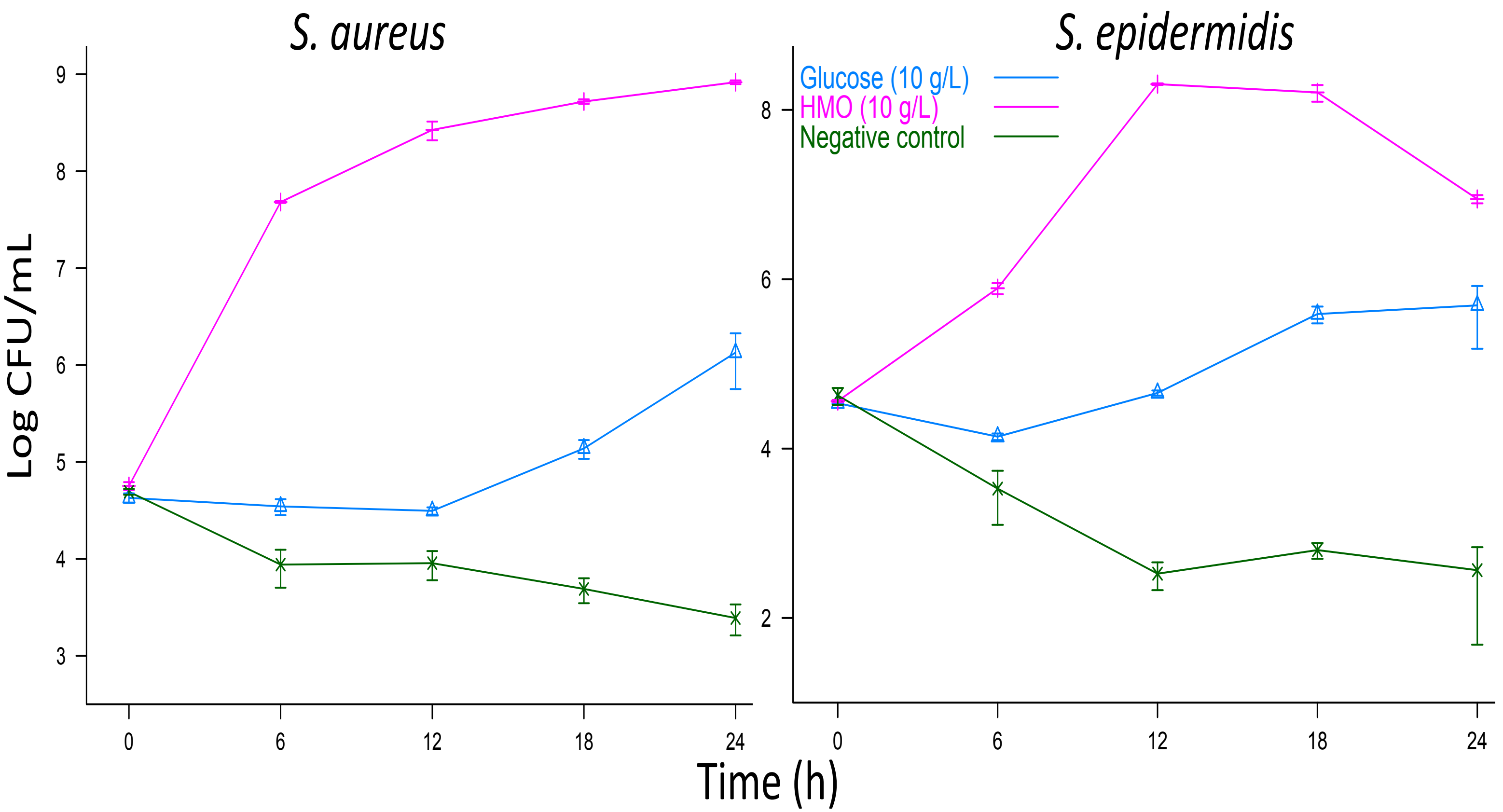


Figure 4. The effects of HMO on the growth of *Staphylococcus* species in minimal media. HMO treatment effect significant ($P < 0.001$) at 6+ h, data presented as mean ± SEM, $n = 3$

Hunt, Bode, & McGuire, preliminary data

Conclusions

- Healthy babies are consuming bacteria daily either from the skin and from milk (750,000 cfu per day)
  - Formula fed babies are provided little inoculum
- Human milk produced during healthy lactation is not sterile
  - Skin can provide some bacterial communities but numerically would be a minor component (10,000-50,000 bacteria/cm^2)

Conclusions

- Human milk microbial communities are highly personalized and complex
- These communities contain immense bacterial diversity- likely even greater than what was observed in this study
- These communities may play a role in mammary health (mastitis)
- The composition of these communities may be influenced by milk components

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