Microbiota in health and disease:

methods (and reasons) for studying the microbiome

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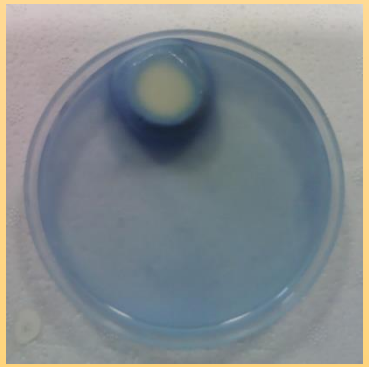
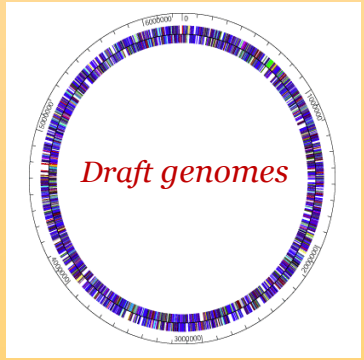
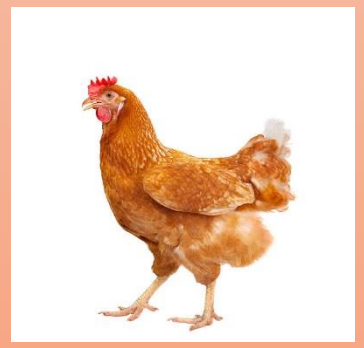
Investigation of spoilage phenotype of *P. fluorescens*





MSc & PhD thesis

Investigation of bacterial, fungal and animal communities in complex environments



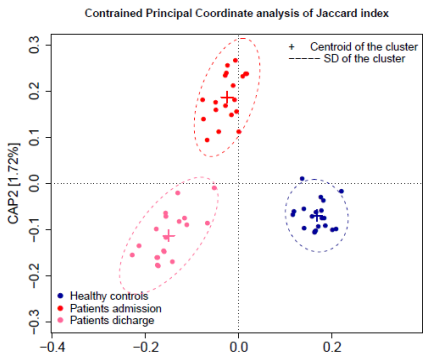
First postdoc

NOW…

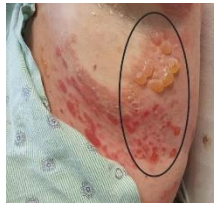
Investigation of bacterial communities in disease

In IBD
- Patients
- Models

In AN
- Patients
- Models



In BP patients
- Gut
- Skin
- mouth



Agenda

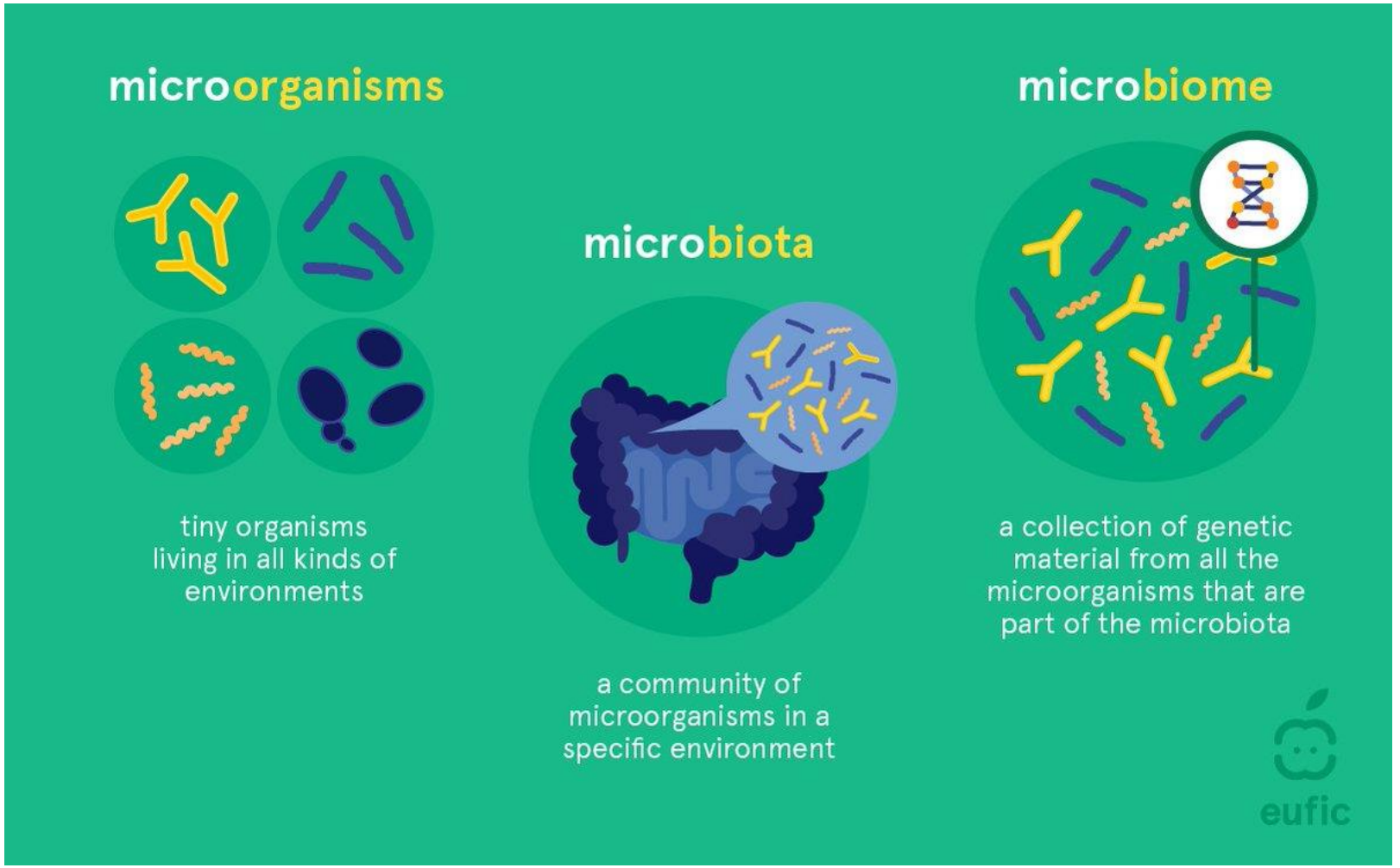
- Microbiome and factors influencing it
- Role of microbiome in health and diseases
- How to study the microbiome

  (with an example of cross-sectional study)
- The gut-brain axis

  (with an example of longitudinal study)
- Animal models and alternative methods to study the microbiome

  (with an example of a model of IBD)

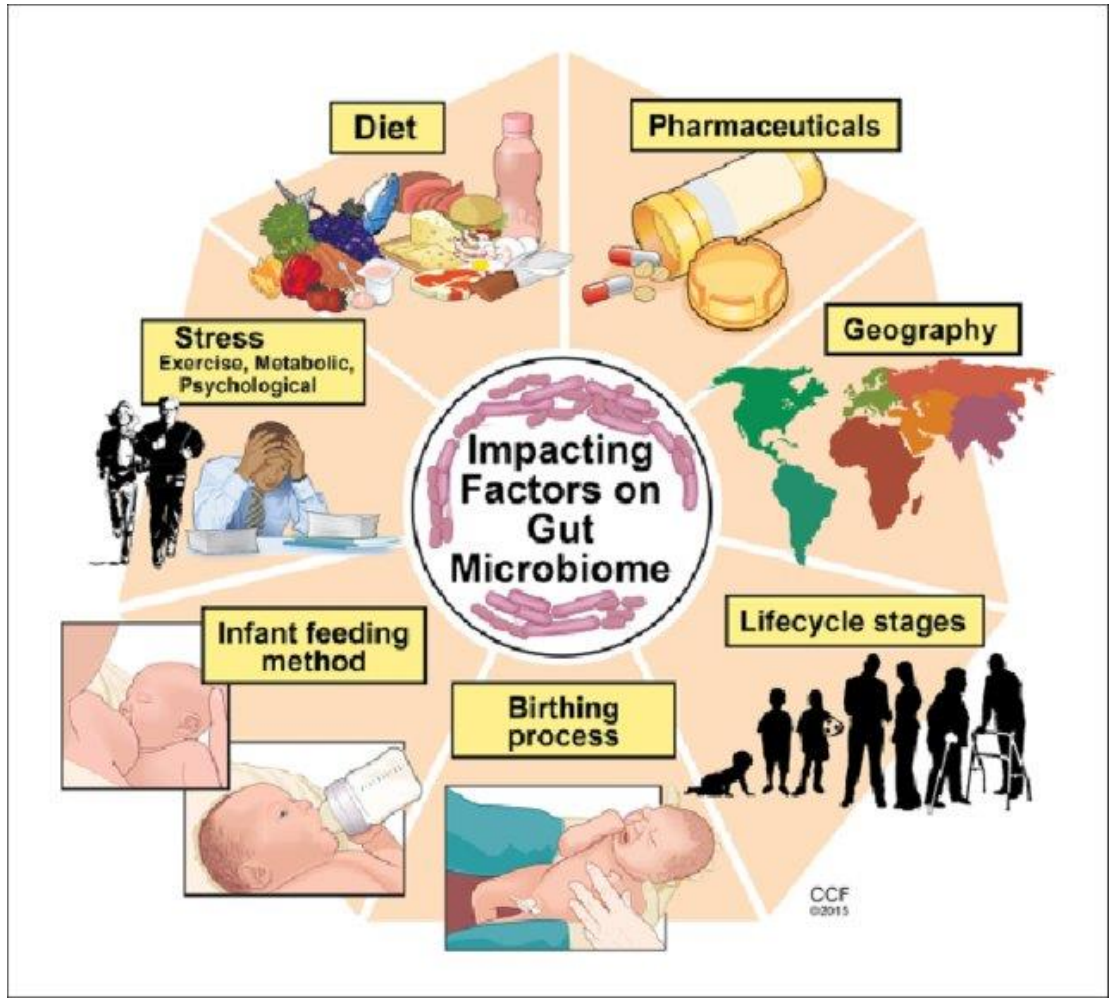
Defining the microbiome (or microbiota?)



Microbiota in numbers

- For years it was believed that human body was harboring bacterial cells in a number that was 10x higher than the human cells
- However, a recent study reported that this ratio is more “1:1”: a 'reference man' (one who is 70 kilograms, 20–30 years old and 1.7 meters tall) contains on average about 30 trillion human cells and 39 trillion bacteria
- An estimated 500–1,000 species of bacteria exist in the human body at any one time, although the number of unique genotypes (subspecies) could be orders of magnitude greater than this.

Factors influencing the human microbiome



From: DOI: 10.1177/0884533615609899

And also…

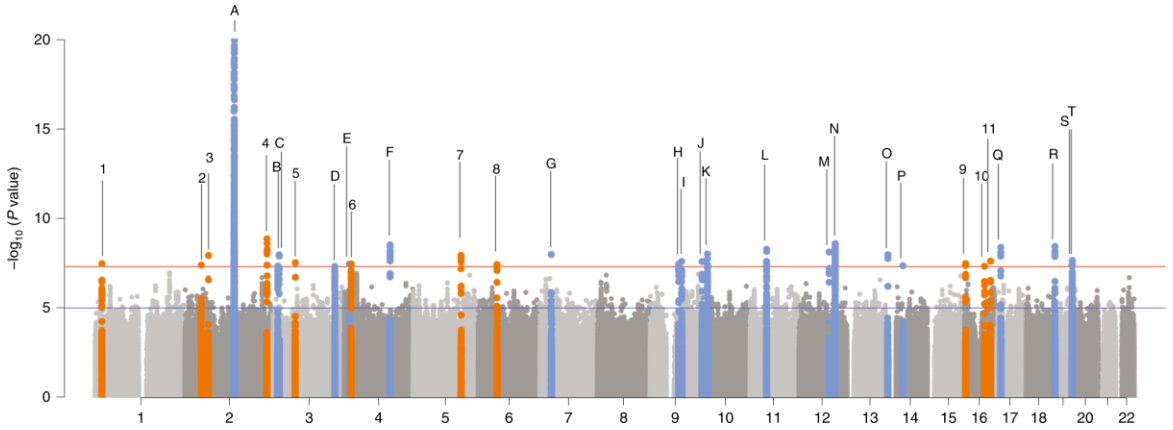
- Human genetics
- Body site
- Lifestyle/occupation
- Circadian rhythm

These factor interact: think of elderly people!
Changes in the microbiome structure of older individuals have often been attributed to altered lifestyles, diets, reduced mobility, decreased immune function, reduced intestinal capability, changed gut morphology, increased use of medication and drugs, and recurrent infections

Human genetic shapes the gut microbiome

How can we determine this?

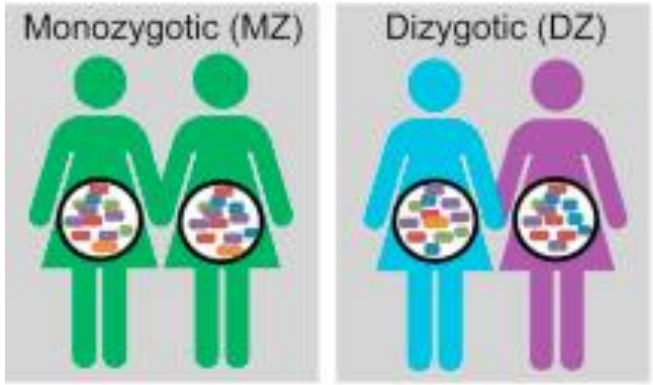
- GWAS (Genome Wide Association Study)



Locus	*P* value	Type	Chr	rsID	Bacteria
A	1.28×10^{-20}	Quant	2	rs182549	Genus *Bifidobacterium*
B	4.66×10^{-8}	Quant	3	rs9864379	Order Gastranaerophilales
C	1.10×10^{-8}	Quant	3	rs75754569	Genus *Peptococcus*
D	4.88×10^{-8}	Quant	3	rs4428215	Family Oxalobacteraceae
E	3.55×10^{-8}	Quant	4	rs10805326	Genus *Intestinibacter*
F	3.06×10^{-9}	Quant	4	rs11098863	Genus *Enterorhabdus*
G	1.04×10^{-8}	Quant	7	rs17159861	Genus group *Eubacterium coprostanoligenes*
H	3.57×10^{-8}	Quant	9	rs602075	Genus *Allisonella*
I	2.57×10^{-8}	Quant	9	rs736744	Genus *Oxalobacter*
J	2.55×10^{-8}	Quant	10	rs12781711	Genus *Ruminococcaceae* UCG013
K	9.80×10^{-9}	Quant	10	rs61841503	Family Peptostreptococcaceae
L	5.29×10^{-9}	Quant	11	rs10769159	Genus *Ruminococcus1*
M	7.57×10^{-9}	Quant	12	rs12320842	Genus *Faecalibacterium*
N	2.58×10^{-9}	Quant	12	rs11110281	Genus *Streptococcus*
O	1.08×10^{-8}	Quant	13	rs7322849	Genus *Bifidobacterium*
P	4.42×10^{-8}	Quant	14	rs8009993	Genus *Ruminococcaceae* UCG009
Q	4.31×10^{-9}	Quant	17	rs7221249	Genus *Erysipelatoclostridium*
R	3.74×10^{-9}	Quant	19	rs67476743	Genus *Tyzzerella3*
S	4.26×10^{-8}	Quant	19	rs830151	Genus *Candidatus Soleaferrea*
T	2.21×10^{-8}	Quant	19	rs35866622	Genus group *Ruminococcus torques*
1	3.34×10^{-8}	Binary	1	rs555115	Genus *Turicibacter*
2	4.07×10^{-8}	Binary	2	rs57440956	Genus *Ruminiclostridium6*
3	1.17×10^{-8}	Binary	2	rs56081657	Genus *Odoribacter*
4	1.42×10^{-9}	Binary	2	rs7574352	Family Peptococcaceae
5	2.98×10^{-8}	Binary	3	rs17066404	Family Peptococcaceae
6	3.53×10^{-8}	Binary	4	rs207281	Genus *Enterorhabdus*
7	1.16×10^{-8}	Binary	5	rs62373497	Genus *Coprococcus1*
8	3.79×10^{-8}	Binary	6	rs9474033	Family Pseudomonadaceae
9	3.34×10^{-8}	Binary	16	rs437867	Genus *Lachnospiraceae* UCG010
10	4.67×10^{-8}	Binary	16	rs17319026	Genus *Anaerostipes*
11	2.42×10^{-8}	Binary	16	rs11647069	Family Bacteroidales S24.7

https://doi.org/10.1038/s41588-020-00763-1

- Twin studies

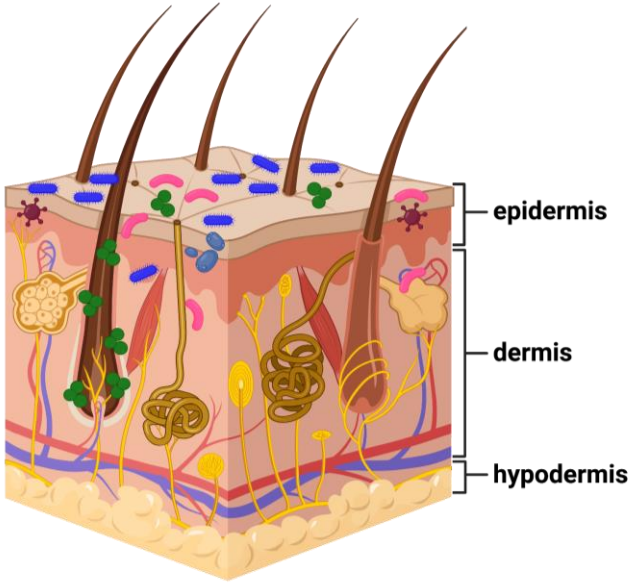


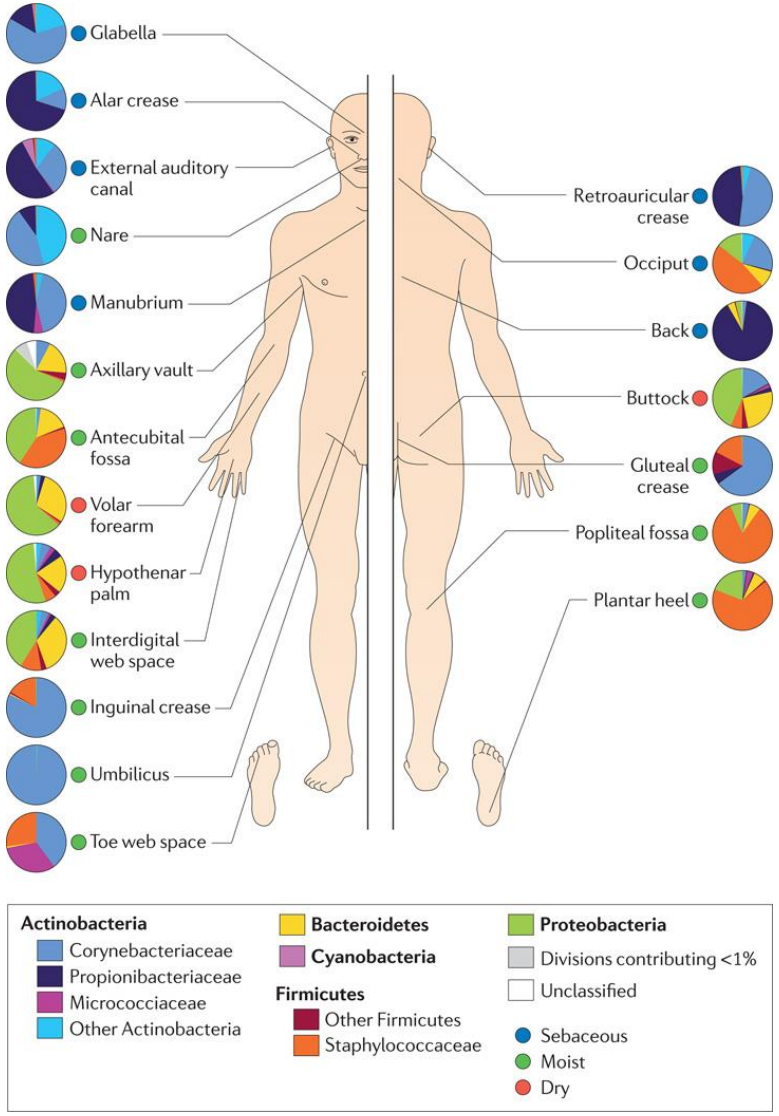
MZ twins have a more similar microbiota than DZ twins

https://doi.org/10.1016/j.cell.2014.09.053

- Different body parts have different microbiome

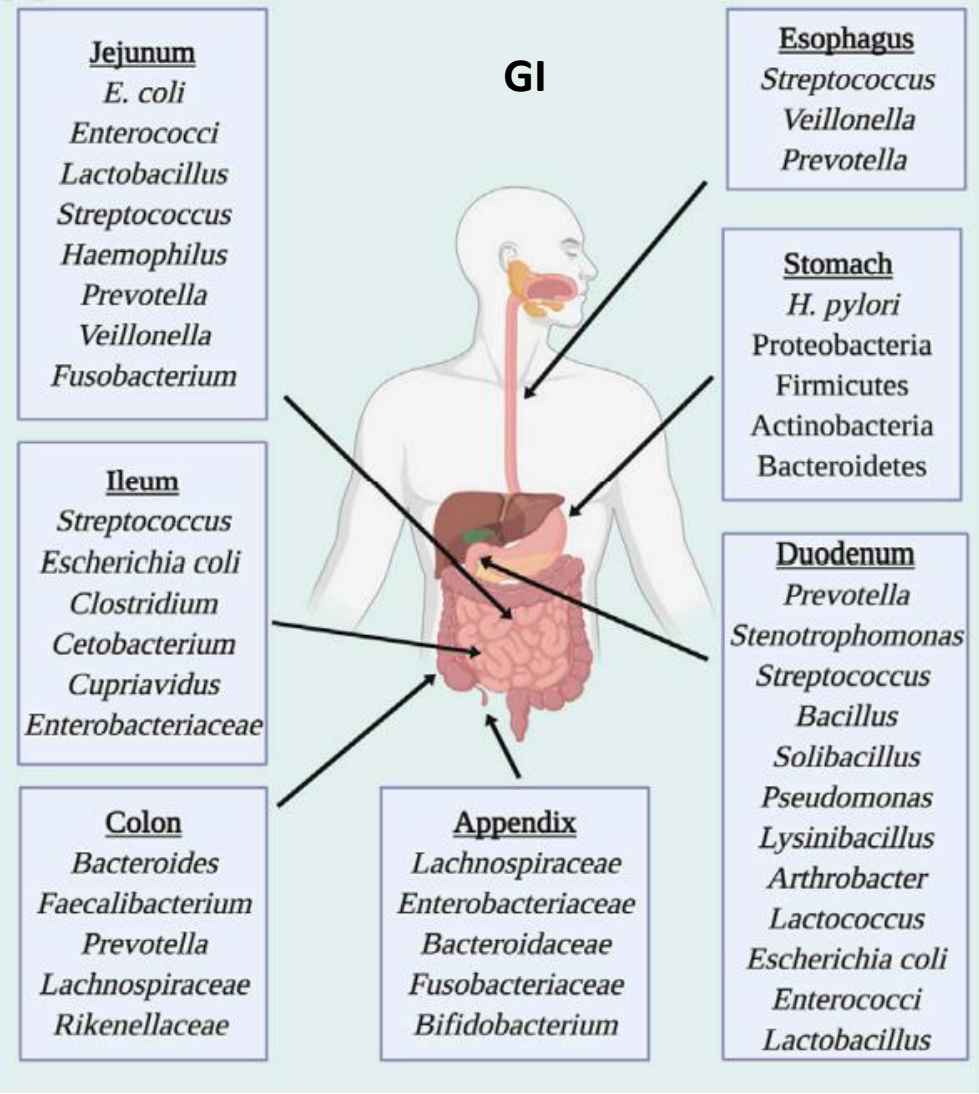
Skin is an ecosystem





doi: 10.1111/j.1753-4887.2012.00493.x

- Different body parts have different microbiome

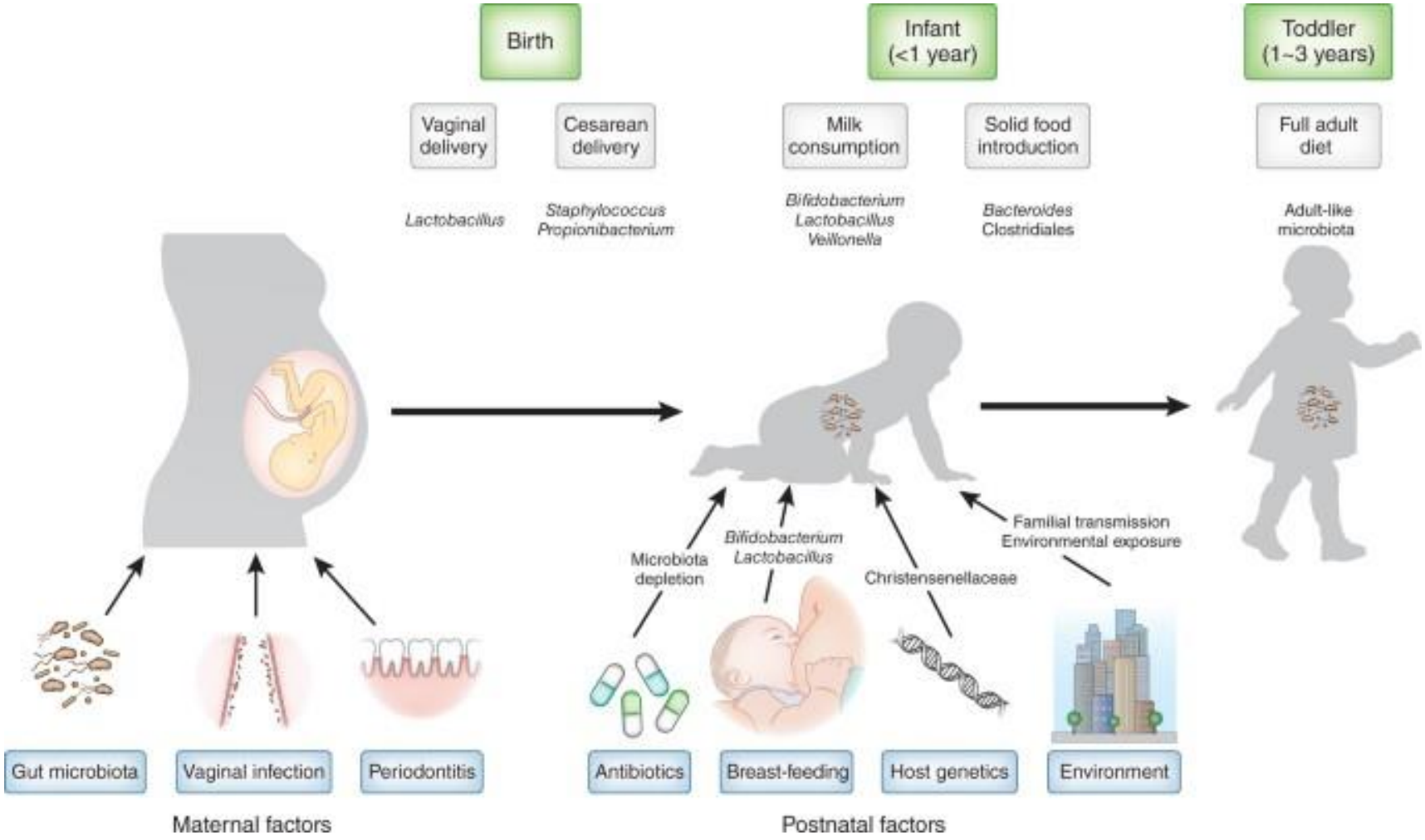


doi: 10.1111/j.1753-4887.2012.00493.x

- This is true also for Gut microbiome.
- Studies report that GI microbiome is quite stable after the first 3 years after birth.

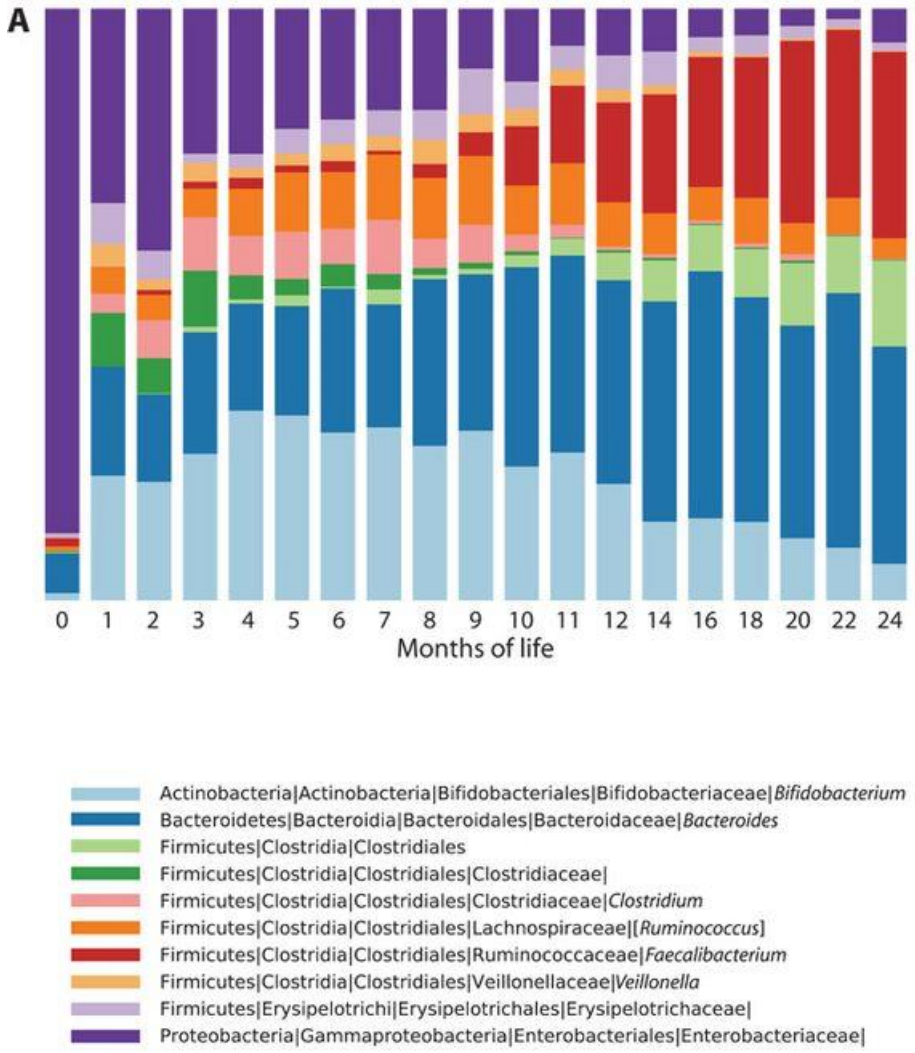
- Different parts of the GI have different conditions (digested food/pH/temperature/oxygen/IS cells).

Microbiome colonization and development



https://doi.org/10.1038/nm.4142

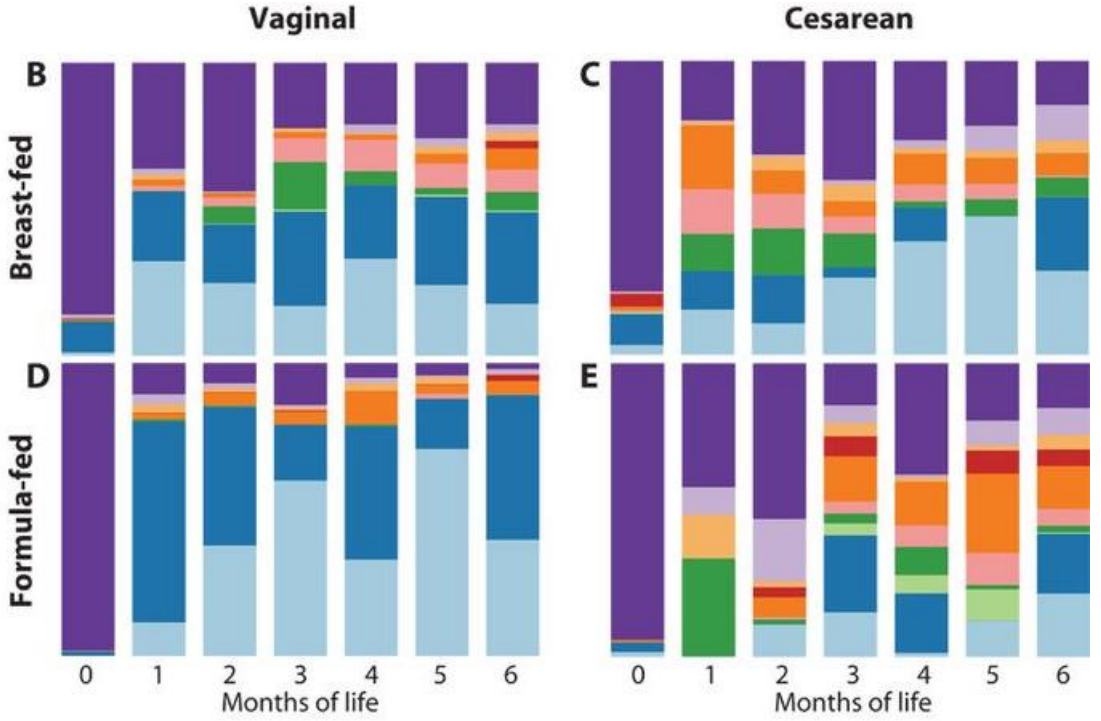
Microbiome colonization and development



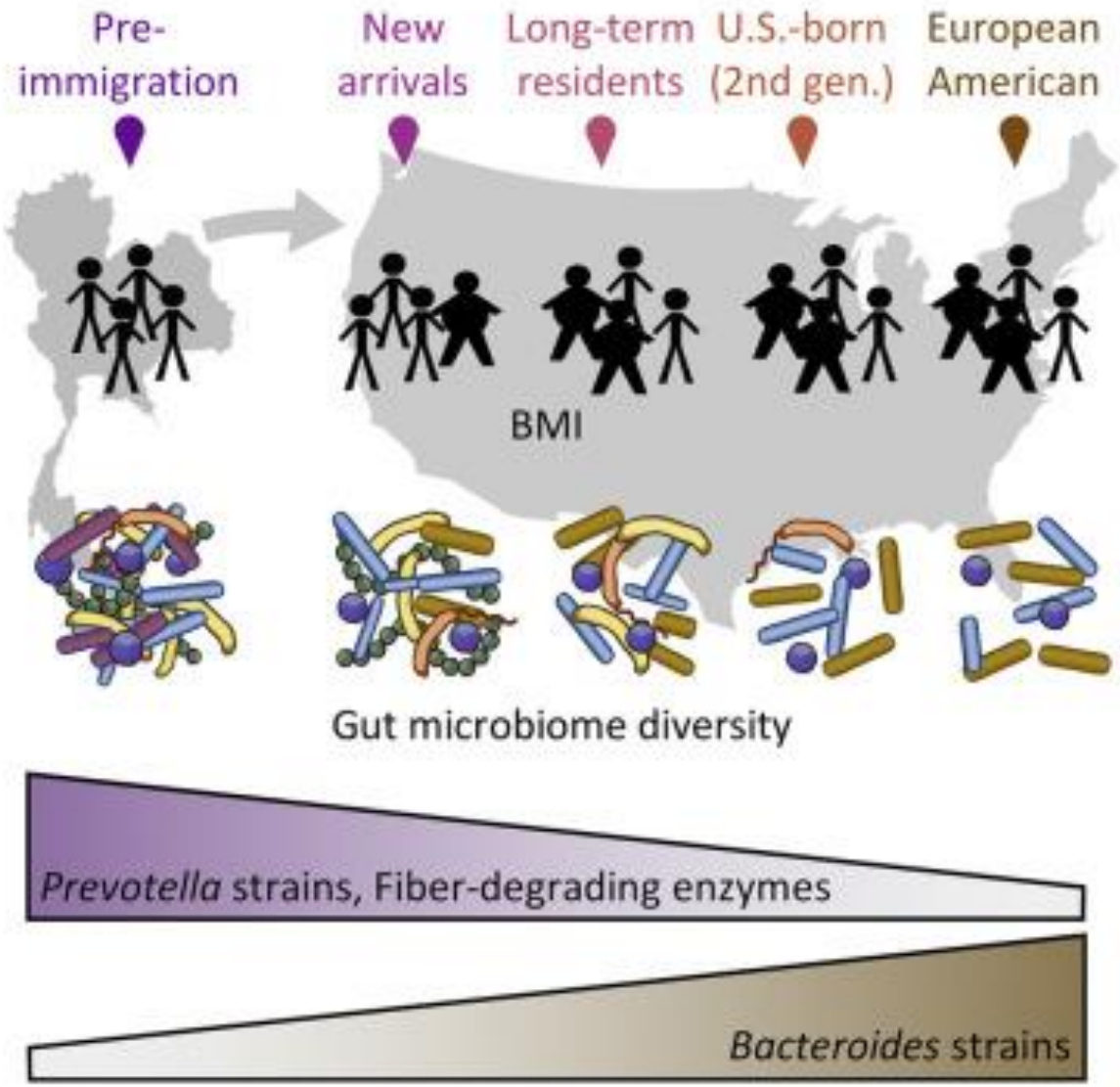
DOI: 10.1126/scitranslmed.aad7121

Microbiome composition is affected by:

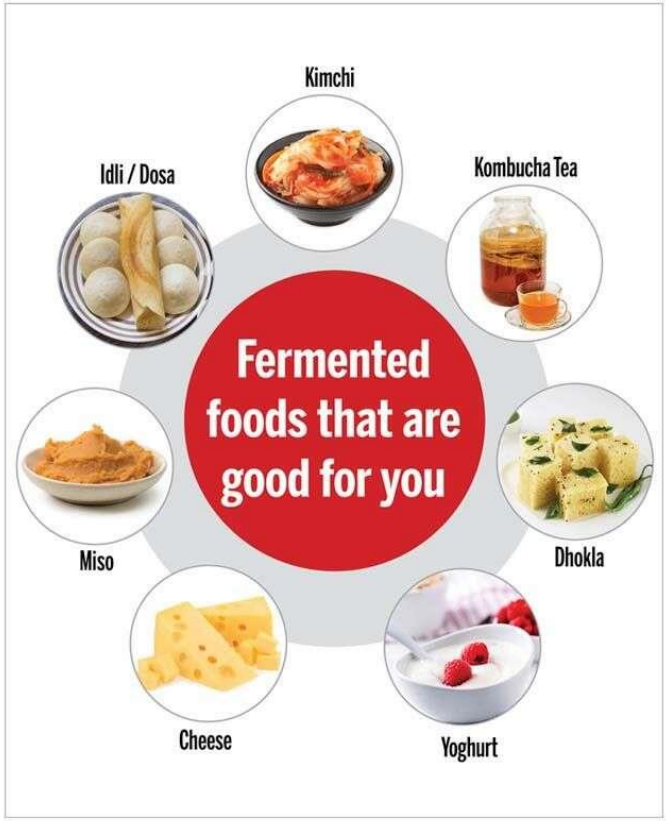
- Method of delivery
- Feeding method



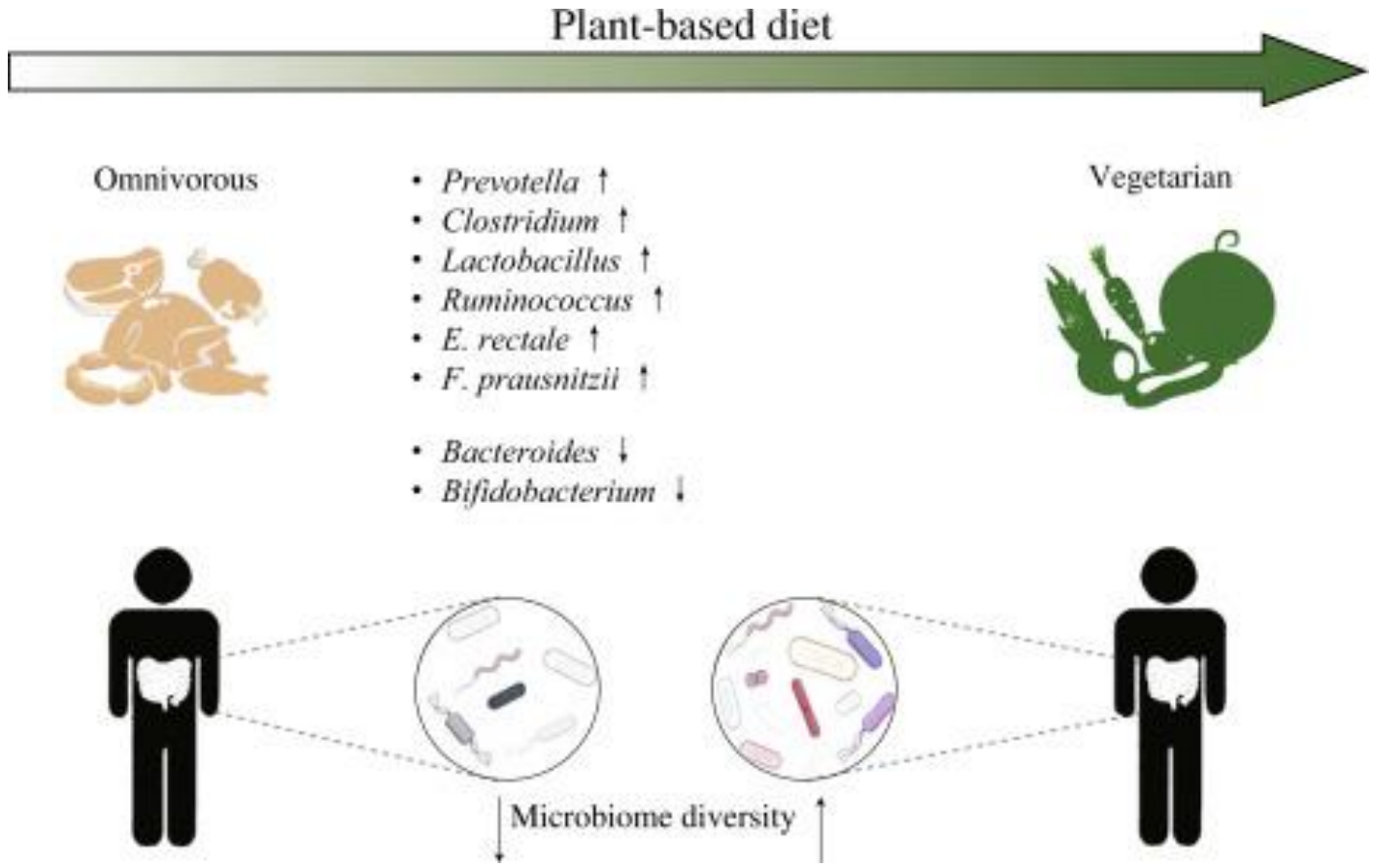
- Diet influences the microbiome



- changing the diet of immigrants from Asia to the United States is linked to an immediate and intense change in the microbiome structure with an impact on their health and development of obesity and its associated diseases
- n=514



Fermented food are associated with beneficial increased diversity and reduced inflammation biomarkers.



- plant-based diet promotes the development of **more diverse and stable** microbial systems.
- vegans and vegetarians have a distinctive microbiome.
- Polyphenols, also abundant in plant foods, increase Bifidobacterium and Lactobacillus, which provide anti-pathogenic and anti-inflammatory effects and cardiovascular protection.
- High fiber intake also encourages the growth of species that ferment fiber into metabolites as short-chain fatty acids (SCFAs with positive health effects, such as improved immunity against pathogens, blood–brain barrier integrity, provision of energy substrates, and regulation of critical functions of the intestine).

https://doi.org/10.1016/j.fshw.2021.11.002

- Drugs influences the microbiome

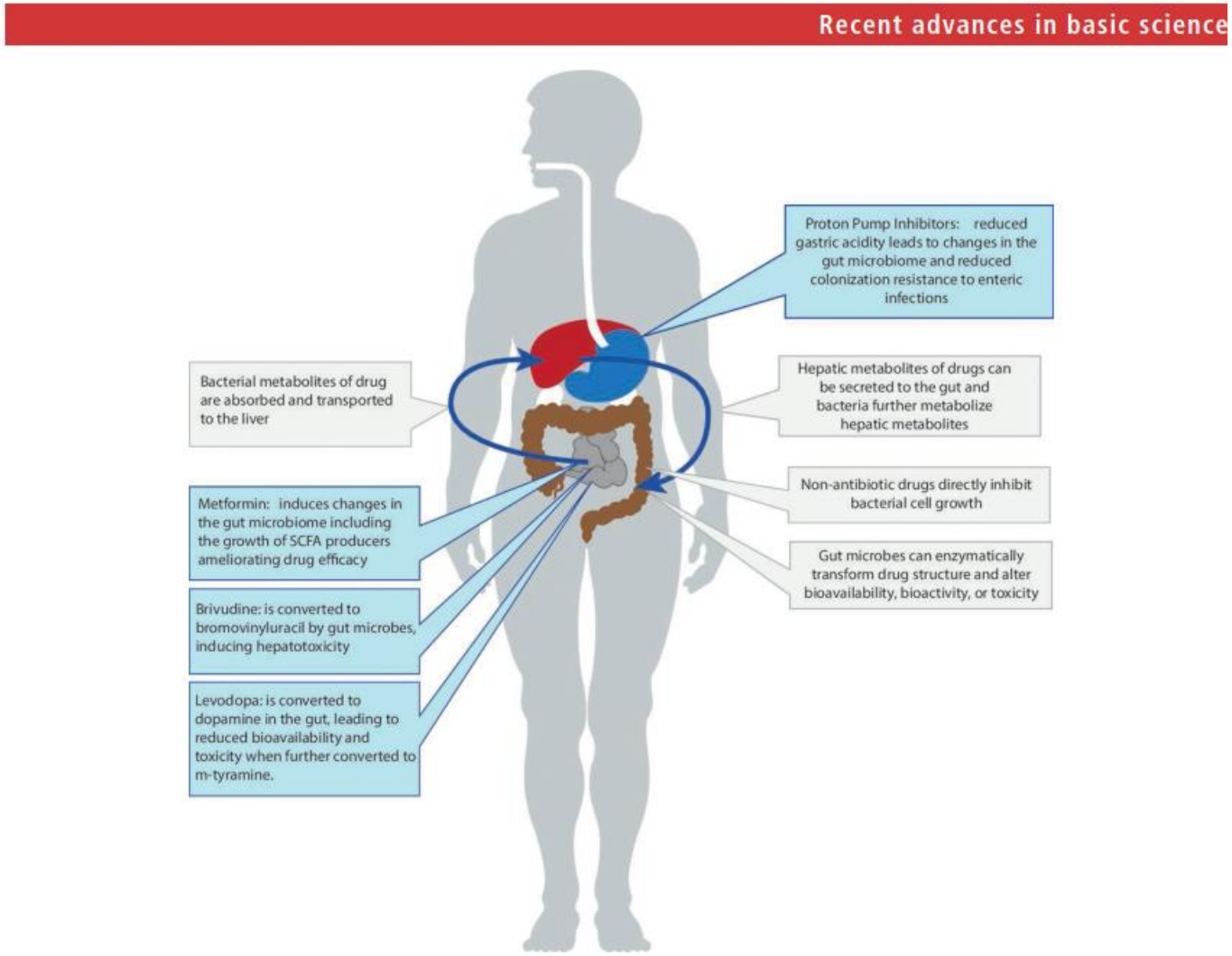
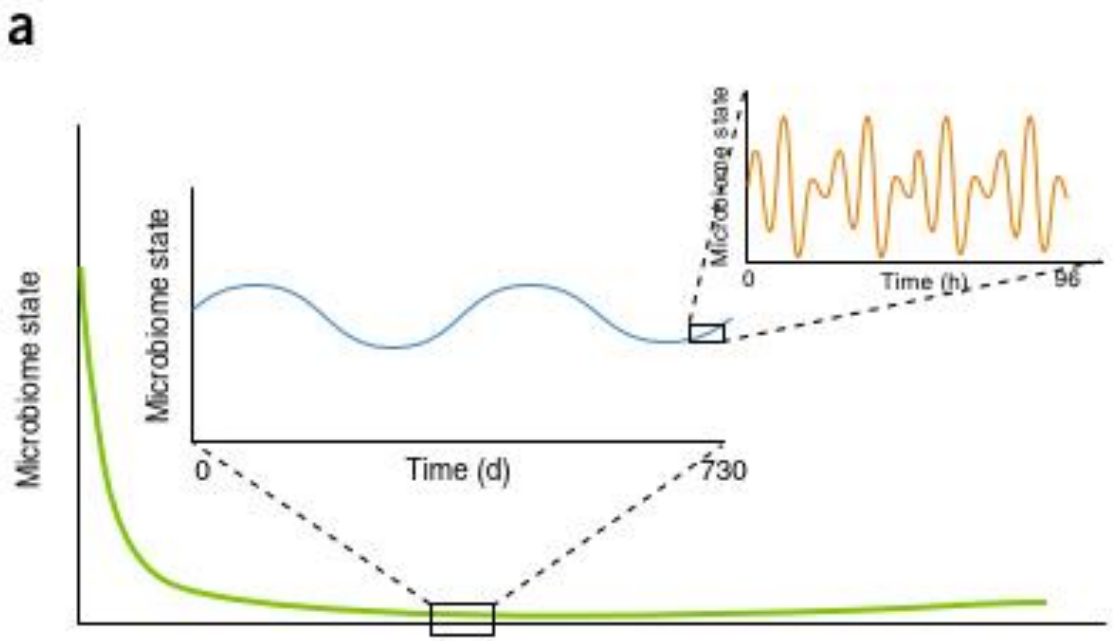


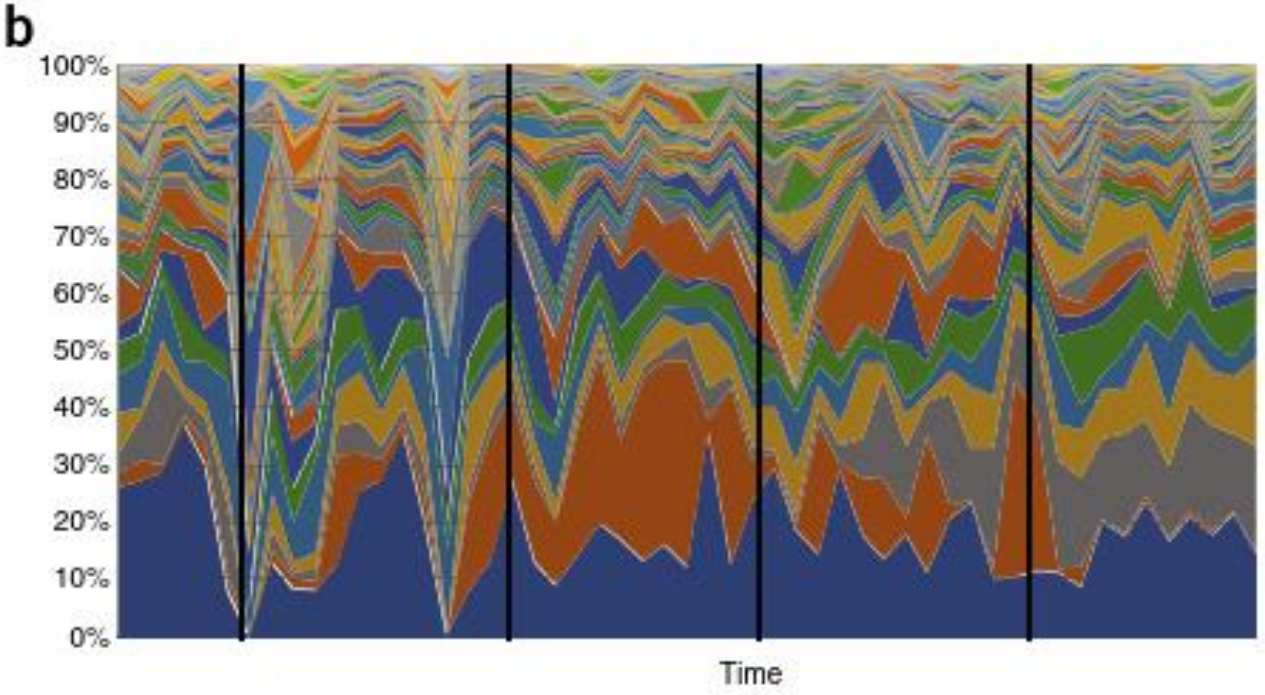
Figure 1 Schematic overview of different interactions between the gut microbiome and commonly used non-antibiotic drugs. SCFA, short-chain fatty acids.

- Stress influences the microbiome
  - Studies show that social stress exposure decreases the abundance of microbes with anti-inflammatory activity such as Bacteroides taxa > which in turn decreases microbial anti-inflammatory metabolites such as SCFAs and contribute to a higher level of inflammation.
  - Combined stress and infection or other inflammatory diseases worsen the outcome of the disease compared to non-stressed subjects.
  - Consuming microbes known for anti-inflammatory activity might be beneficial for people with anxiety disorder and unmanageable stress levels.

Circadian variation of microbiome

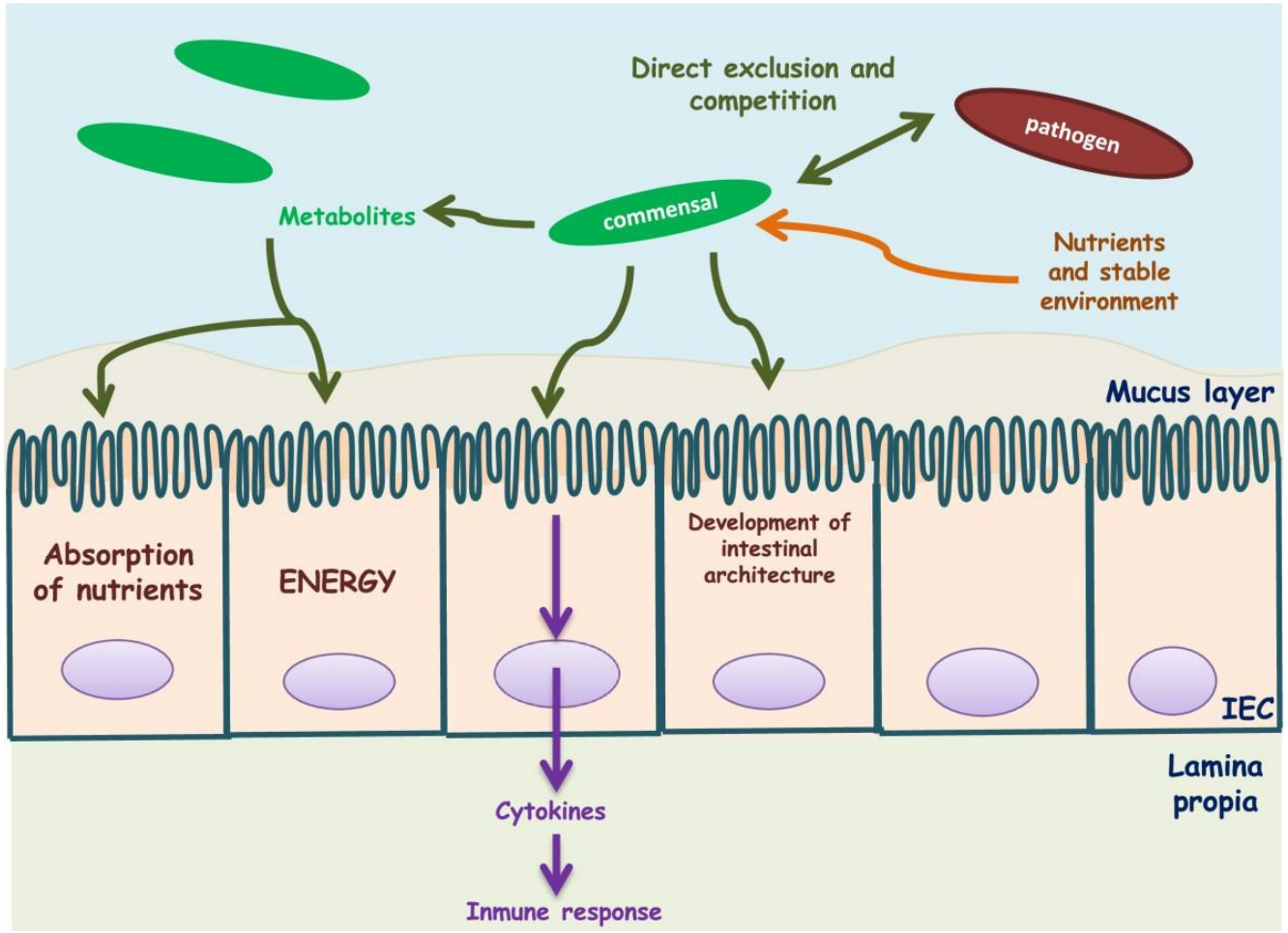
Circadian= follows a 24-hour cycle

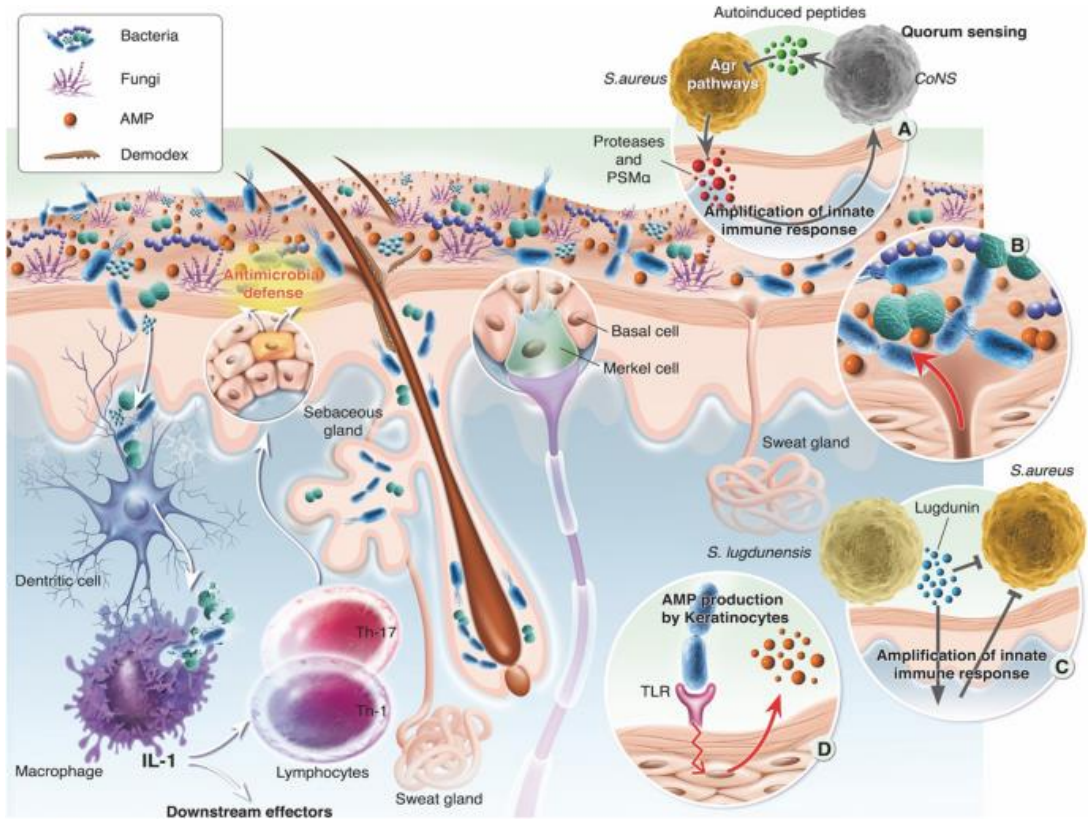




doi:10.1038/nm.4517

- A healthy microbiome has a beneficial role on the host

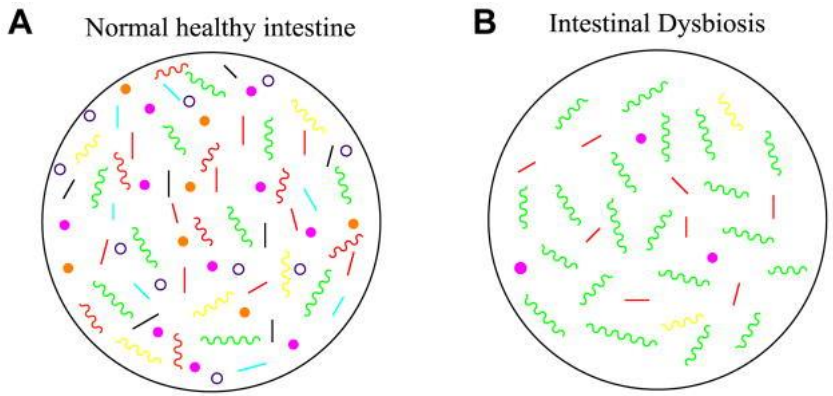




https://doi.org/10.1186/1475-2859-12-71

https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-021-01062-5

A shift from the healthy (balanced) microbiome composition is called dysbiosis: this could be either abnormal composition or reduced or enhanced biodiversity.



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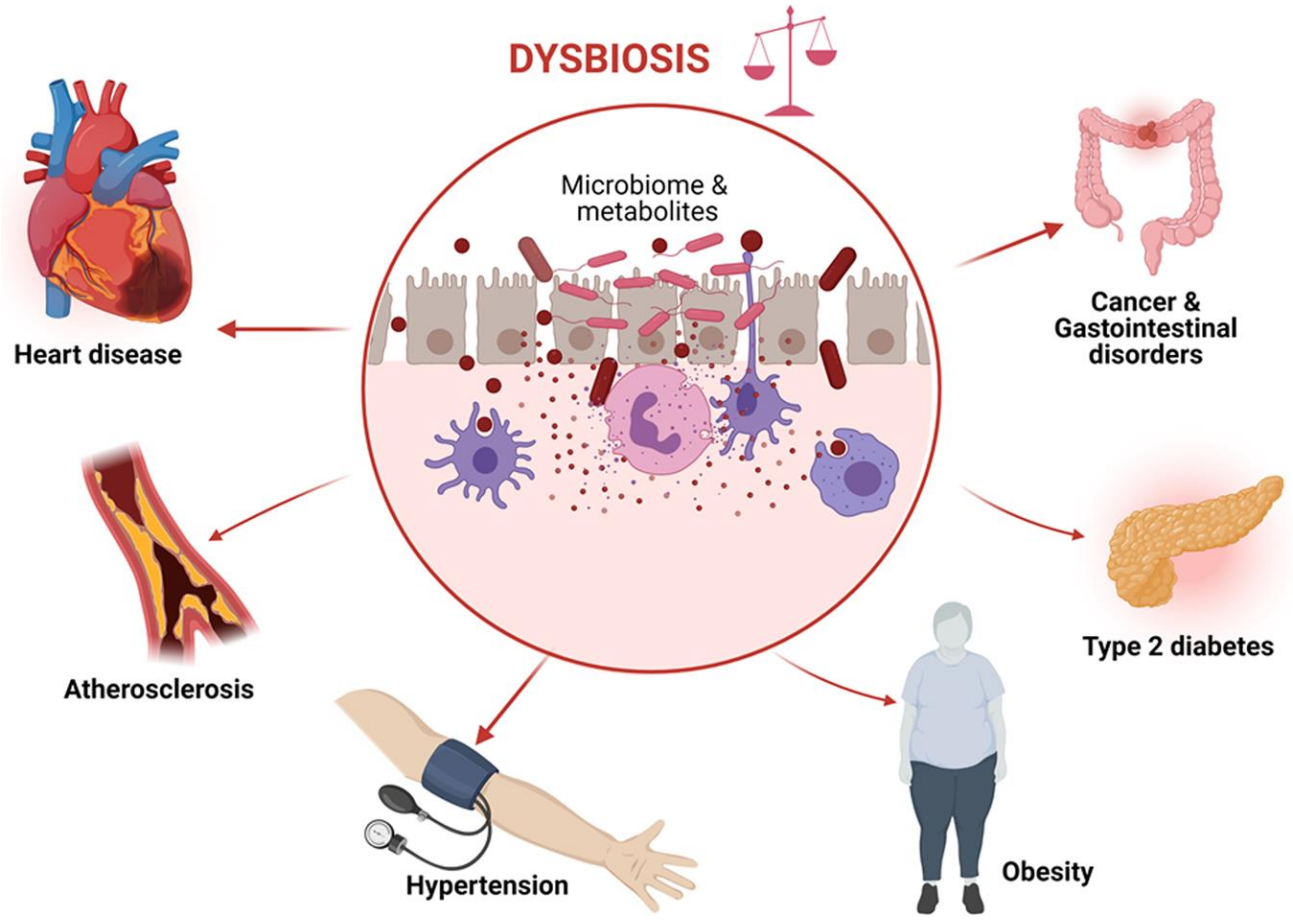

doi: 10.1097/MIB.0000000000000750
https://doi.org/10.1038/s41371-022-00698-6

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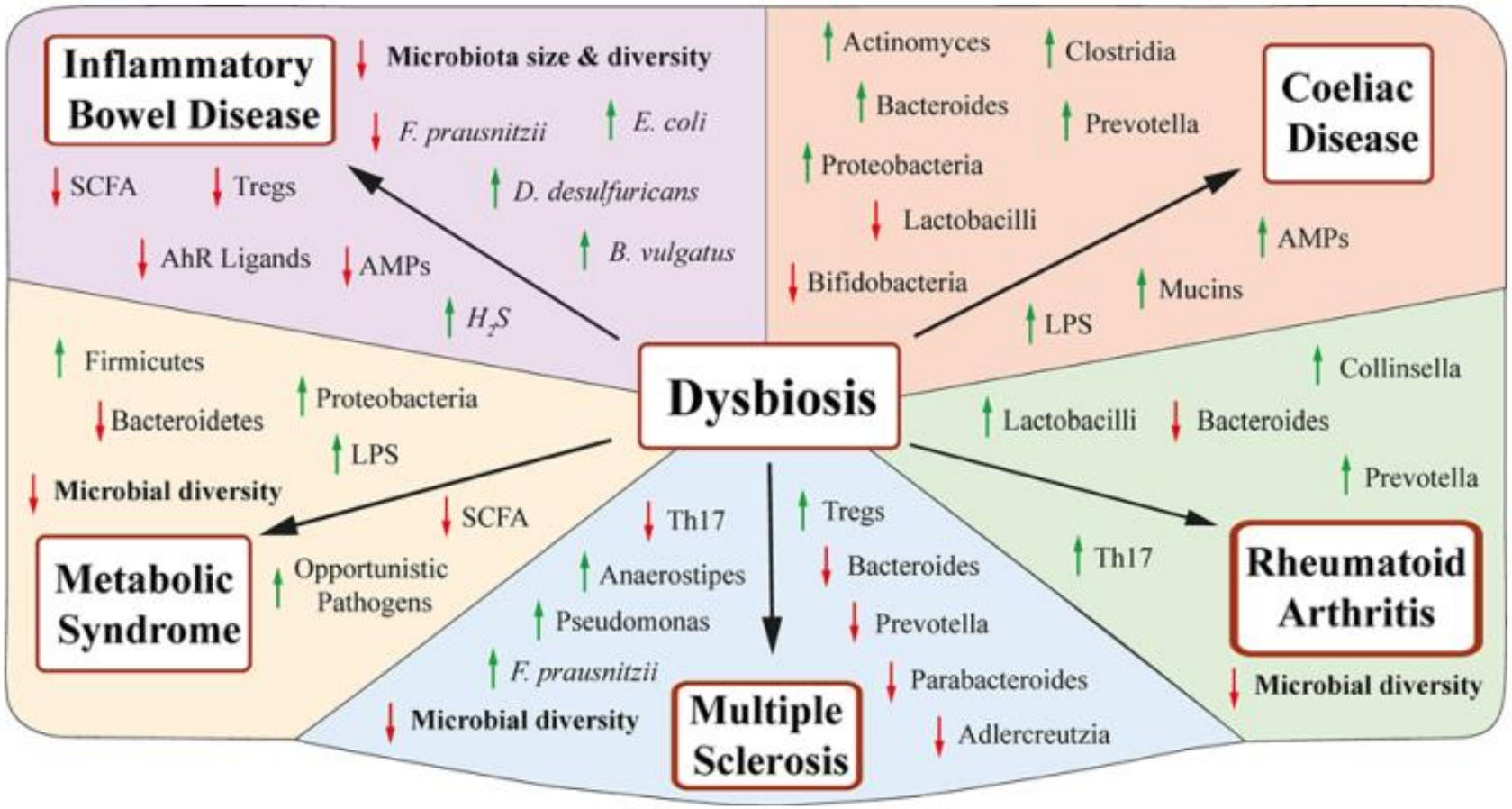
https://doi.org/10.1042/BSR20203850



Body site	Main taxa in healthy individuals	Main alterations in disease	Associated diseases	References
Vagina	*Lactobacillus crispatus*, *L. iners*, *L. gasseri*, *L. jensenii Streptococcus*, *Bifidobacterium*	↑ *Sneathia*, *Atopobium*, *Gardnerella*	Bacterial vaginosis, Vulvovaginal infections (RVVI), HPV infections and cervical cancer	Felten et al. (1999), Zhou et al. (2007), Di Paola et al. (2017)
	Very low abundance of anaerobes, *Prevotella*, *Atopobium*, Sneathia Gardnerella	↓ Lactobacilli	Symptoms associated with these include discomfort, odor, discharge, infertility, and, if pregnant, could even lead to miscarriages	
Skin	*Staphylococcus*, *Propionibacterium*, *Corynebacterium*, and *Streptococcus*	↑ *S. aureus*, *S. epidermidis*, *P. acnes*, Proteobacteria	Psoriasis, atopic dermatitis, systemic lupus erythematosus and alopecia	Chang et al. (2018), Ho et al. (2019), Paller et al. (2019), Bay et al. (2020), Huang et al. (2020)
		↓ *Acinetobacter Cutibacterium*, *Propionibacterium*, *Corynebacterium*, and *Staphylococcus*		
Eye	*Staphylococcus*, *Propionibacterium*, and *Pseudomonas*	↑*Delftia* and *Bacteroides*	Keratoconjunctivitis, mucosa-associated lymphoid tissue (MALT) lymphoma, and high glucose levels on the ocular surface due to diabetes	Asao et al. (2019), Li et al. (2019), Suzuki et al. (2020)
		↓Proteobacteria and Acinetobacter		
Ear	*Corynebacterium*, *Staphylococcus*, and *Propionibacterium*	↑ *Haemophilus*, *Alloiococcus Staphylococcus*, *Turicella*, *Moraxella*, *Streptococcus* and *Stenotrophomonas*	Otitis media infections: Acute Otitis Media (AOM) or Chronic Otitis Media with Effusion (COME)	Lappan et al. (2018), Jervis-Bardy et al. (2019), Kolbe et al. (2019)
Nasopharyngeal tract	*Corynebacteriaceae*, *Staphylococcaceae*, *Peptoniphilaceae*, *Carnobacteriacea*, *Staphylococcus*, *Corynebacterium*, *Alloiococcus*, *Haemophilus*, *Streptococcus*, *Granulicatella*, and *Moraxella*	↑ *Streptococcus*, *Haemophilus*, *Moraxella*, Proteobacteria, *Escherichia*, *Roseateles*, and *Pseudomonas*	Asthma, influenza A virus (IAV), bronchiolitis, and rhinosinusitis acute respiratory illness (ARI)	Teo et al. (2015), Stewart et al. (2017), Copeland et al. (2018), Wen et al. (2018), Kang and Kang, (2021)
		↓*Corynebacterium*, *Moraxella* and *Dolosigranulum*		
Oral	*Streptococcus*, *Gemella*, *Abiotrophia*, *Granulicatella*, *Rothia*, *Neisseria*, and *Prevotella*	↑ *Porphyromonas*, *Tannerella*, *Prevotella*, *Filifactor*	Dental cavities, gingivitis, periodontitis, oral cancer	Dewhirst et al. (2010), Crielaard et al. (2011), Huang et al. (2011), Kennedy et al. (2019), Sulyanto et al. (2019)
Gastrointestinal tract	*Clostridium*, *Bacteroides*, *Lactobacillus*, *Coprobacillus*, *Escherichia/Shigella*, *Bifidobacterium*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Akkermansia muciniphila*, *Enterococcus*, *Streptococcus*, *Veillonella*, *Prevotella*, *Helicobacter pylori*, *Stenotrophomonas*, *Lactococcus*, *Bacillus*, *Solibacillus*, *Pseudomonas*, *Arthrobacter*, *Lysinibacillus*	↑ *Veillonella*, *Fusobacterium*, *Prevotella* and *Gemella*, *Parvimonas* and other Proteobacteria	Gastroesophageal reflux disease (GERD), Barrett's esophagus, or esophageal carcinoma, appendicitis	Pei et al. (2004), Maldonado-Contreras et al. (2011), Zoetendal et al. (2012), Guinane et al. (2013), Liu et al. (2013), Khan et al. (2014), Angelakis et al. (2015), Sundin et al. (2017), Gong et al. (2019), Fan et al. (2020), James et al. (2020)
		↓ *Bacteroides*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila* and Spirochaetes		

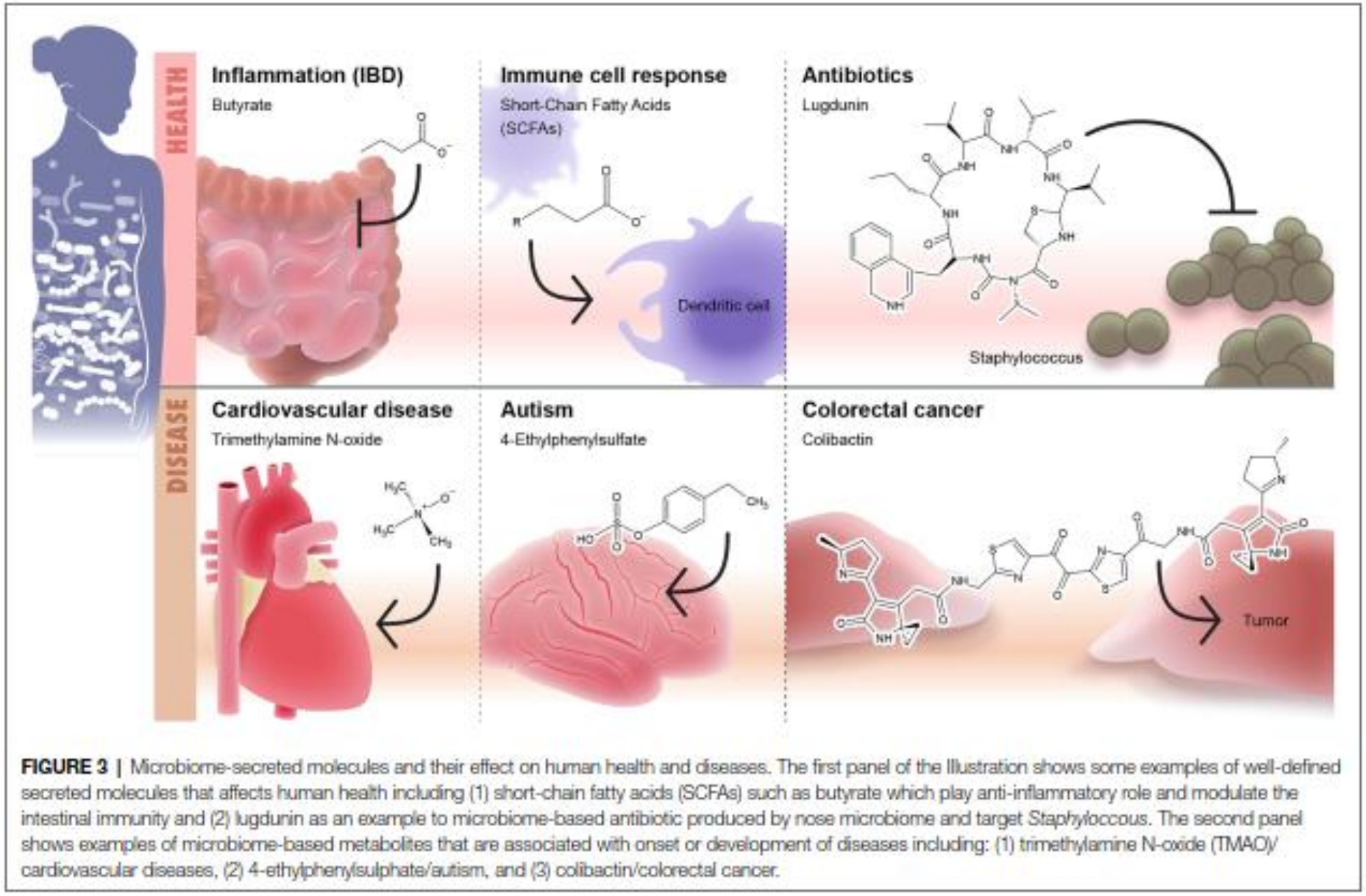
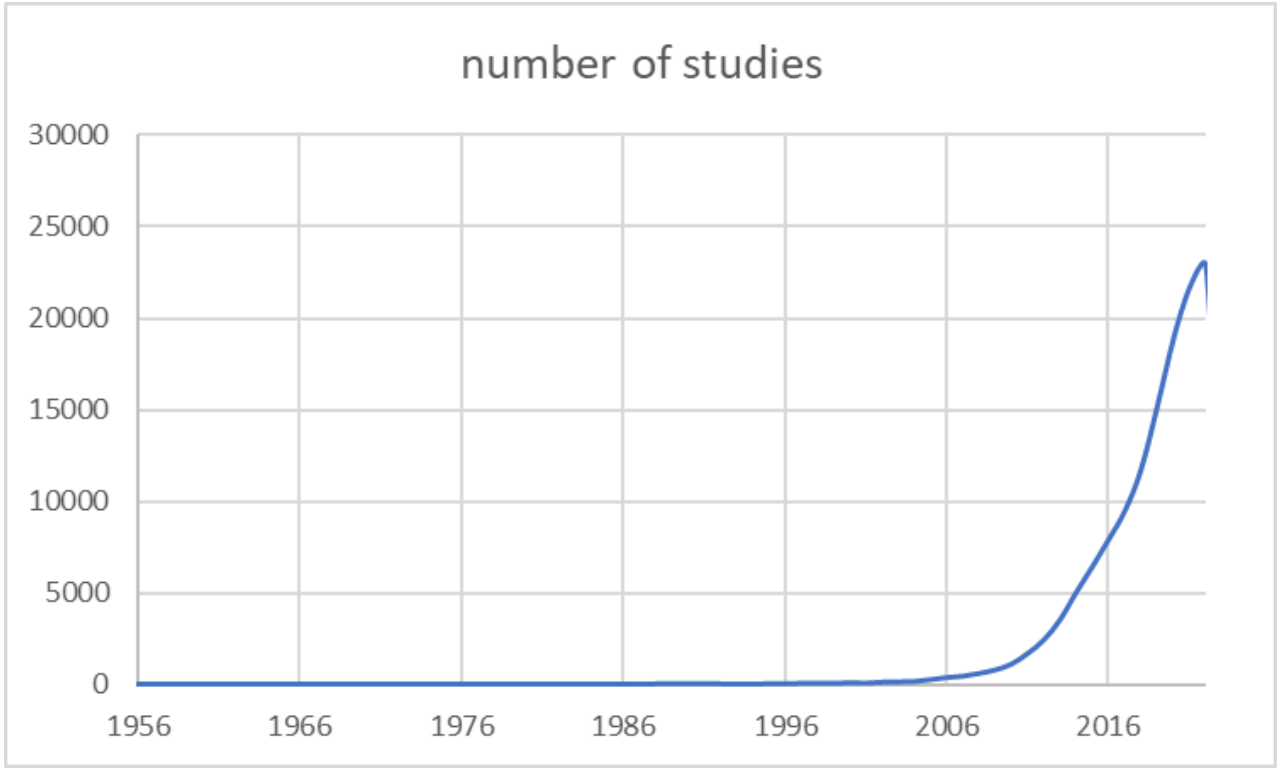


FIGURE 3 | Microbiome-secreted molecules and their effect on human health and diseases. The first panel of the Illustration shows some examples of well-defined secreted molecules that affects human health including (1) short-chain fatty acids (SCFAs) such as butyrate which play anti-inflammatory role and modulate the intestinal immunity and (2) lugdunin as an example to microbiome-based antibiotic produced by nose microbiome and target *Staphylococcus*. The second panel shows examples of microbiome-based metabolites that are associated with onset or development of diseases including: (1) trimethylamine N-oxide (TMAO)/ cardiovascular diseases, (2) 4-ethylphenylsulphate/autism, and (3) colibactin/colorectal cancer.

- The human **microbiota** consists of the 10-100 trillion symbiotic microbial cells harbored by each person, primarily bacteria in the gut
- The human **microbiome** consists of the genes these cells harbor.

The research in human microbiome has been invested in a greater interest in recent years as testified by the number of studies looking at it.

But what happened around year 2000 that caused this sudden rise?



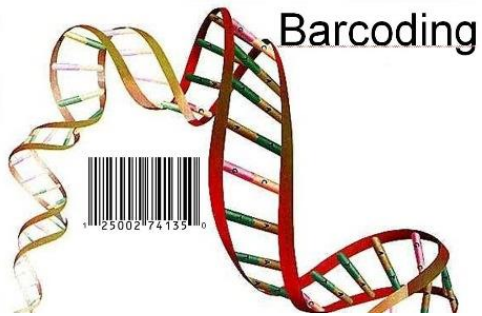
doi: 10.1111/j.1753-4887.2012.00493.x

Before 2000s: **culture-dependent approaches**

- Some microbes can be grown in vitro by using specific growth media and then identified through microscopy.
- Problem is that only a tiny percentage of microorganisms can grow in artificial conditions.

- The percentage of unculturable bacteria varies based on the biota that is investigated [99% of the bacteria is unculturable in some soils; 50% in the mouth; unknown for other body sites]

Molecular fingerprinting or barcoding

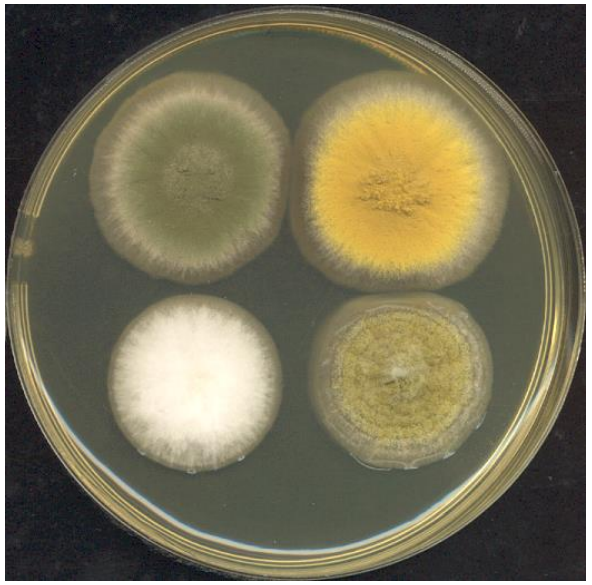


"A tool for rapid species identification based on DNA sequences"

Kress and Erickson, 2008

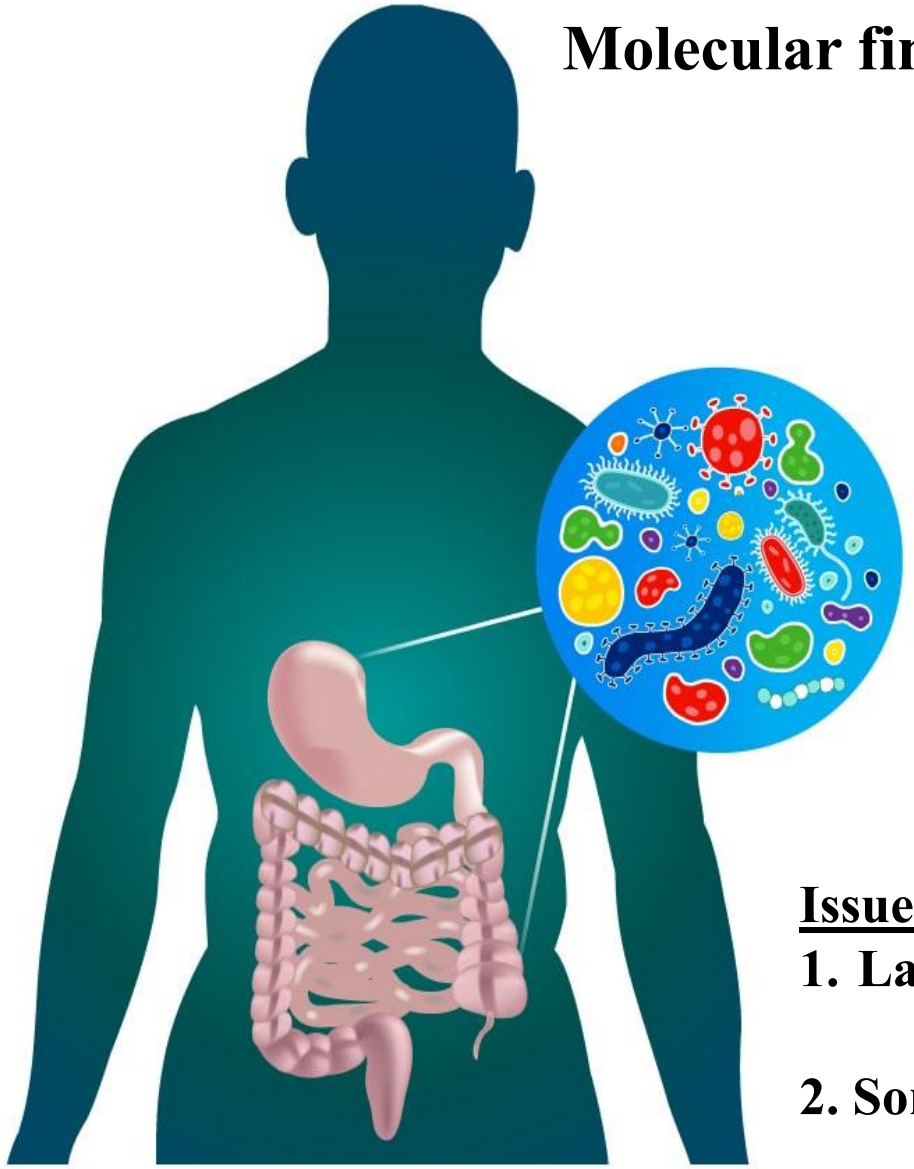
16S to identify bacteria

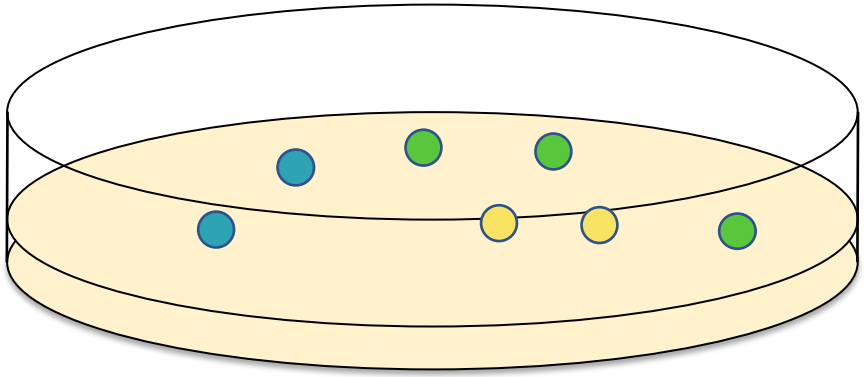
ITS or 18S to identify yeasts



COI to identify animals

Molecular fingerprinting: traditional culture-based approach

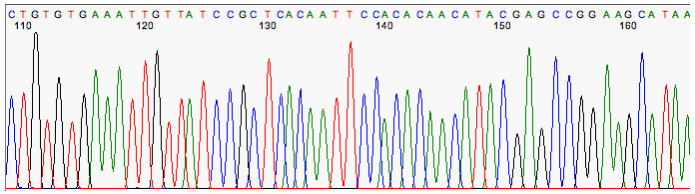




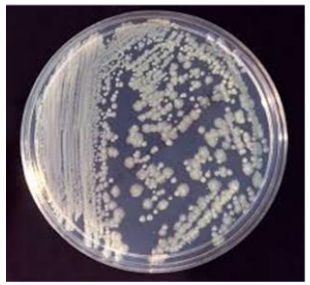
Issues:

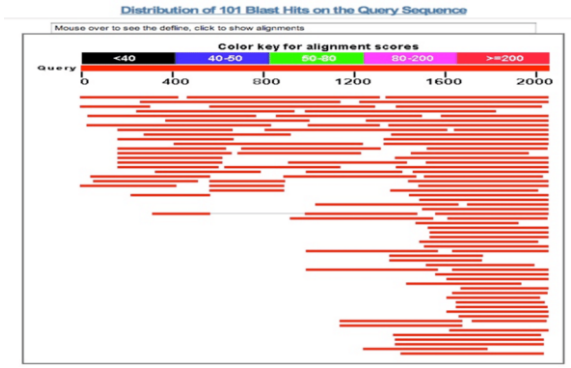
1. **Large sampling error: tens/hundred from 10^6 or more (miss rare species)**
2. **Some species may not grow on artificial media**

Single individual DNA extraction and marker amplification



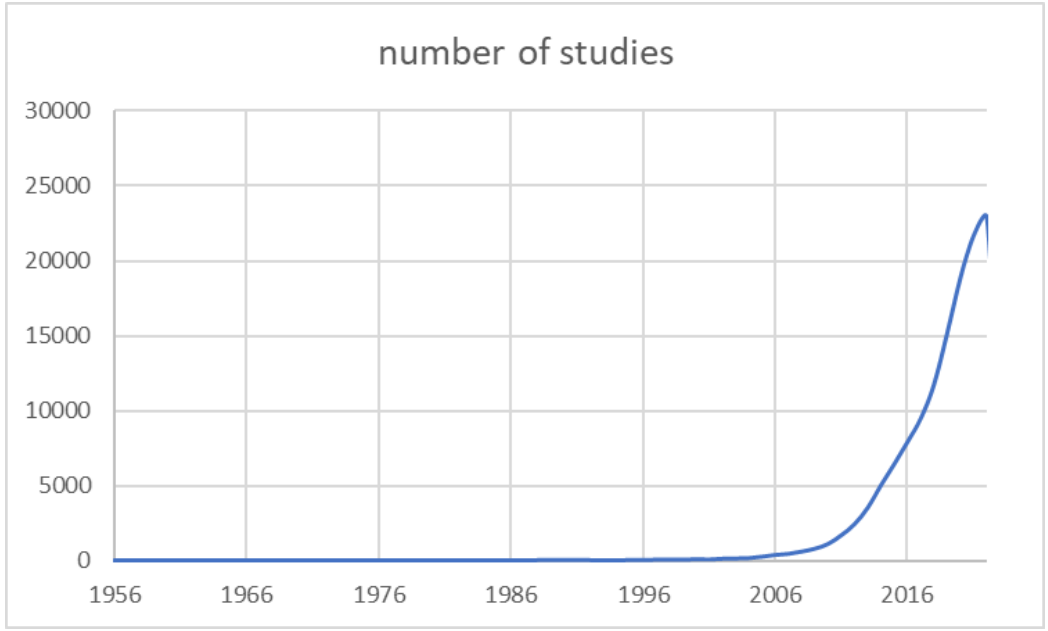
16S to identify bacteria

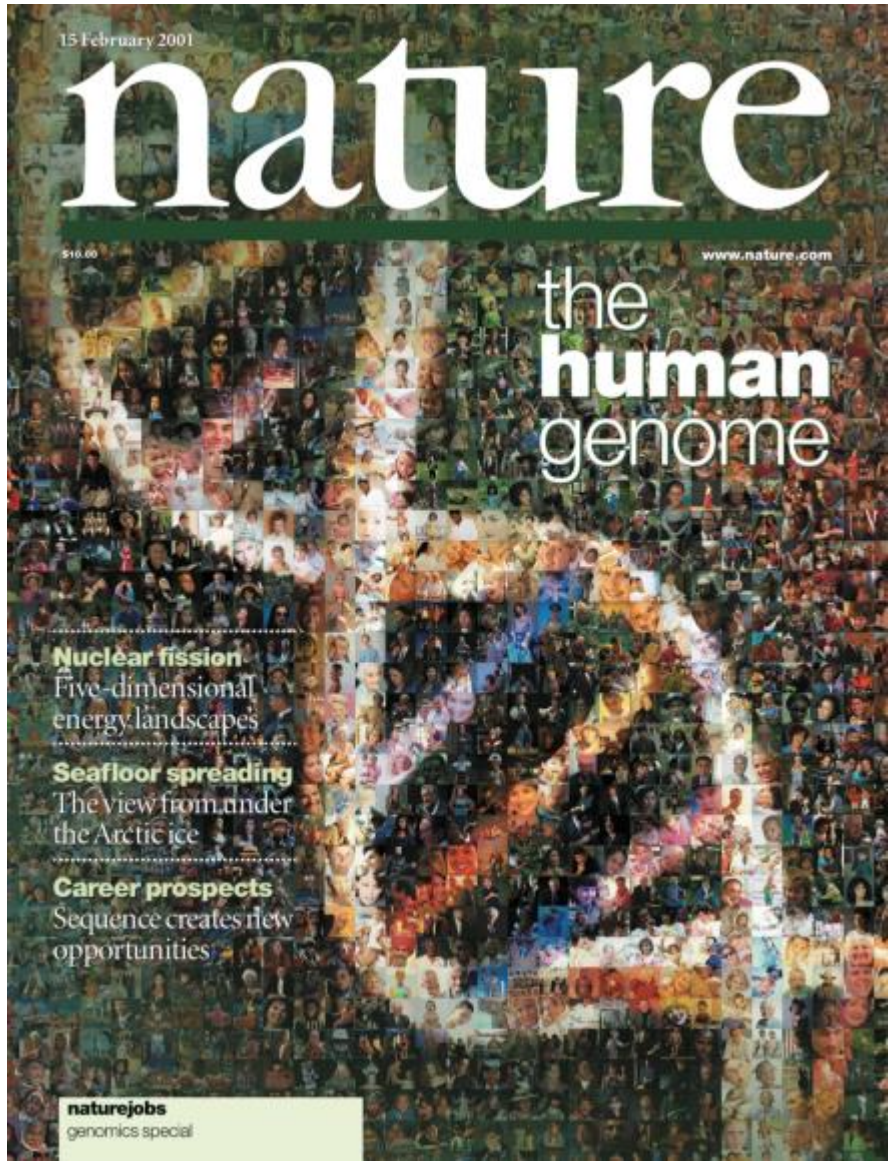




Cost/isolate= 4-5 euros

But what happened around year 2000 that caused this sudden rise?





the Human Genome Project (HGP):

- launched in 1990
- Aim: sequence the 3 billion bases of the human genome.
- Additional goals: generation of physical and genetic maps of the human genome, as well as mapping and sequencing of **key model organisms** used in biomedical research.

Whole-Genome Random Sequencing and Assembly of *Haemophilus influenzae* Rd

Robert D. Fleischmann, Mark D. Adams, Owen White, Rebecca A. Clayton, Ewen F. Kirkness, Anthony R. Kerlavage, Carol J. Bult, Jean-Francois Tomb, Brian A. Dougherty, Joseph M. Merrick, Keith McKenney, Granger Sutton, Will FitzHugh, Chris Fields,* Jeannine D. Gocayne, John Scott, Robert Shirley, Li-Ing Liu, Anna Glodek, Jenny M. Kelley, Janice F. Weidman, Cheryl A. Phillips, Tracy Spriggs, Eva Hedblom, Matthew D. Cotton, Teresa R. Utterback, Michael C. Hanna, David T. Nguyen, Deborah M. Saudek, Rhonda C. Brandon, Leah D. Fine, Janice L. Fritchman, Joyce L. Fuhrmann, N. S. M. Geoghagen, Cheryl L. Gnehm, Lisa A. McDonald, Keith V. Small, Claire M. Fraser, Hamilton O. Smith, J. Craig Venter†

THE *DROSOPHILA* GENOME
REVIEW

The Genome Sequence of *Drosophila melanogaster*

Initial sequencing and comparative analysis of the mouse genome

Mouse Genome Sequencing Consortium*

**articles*

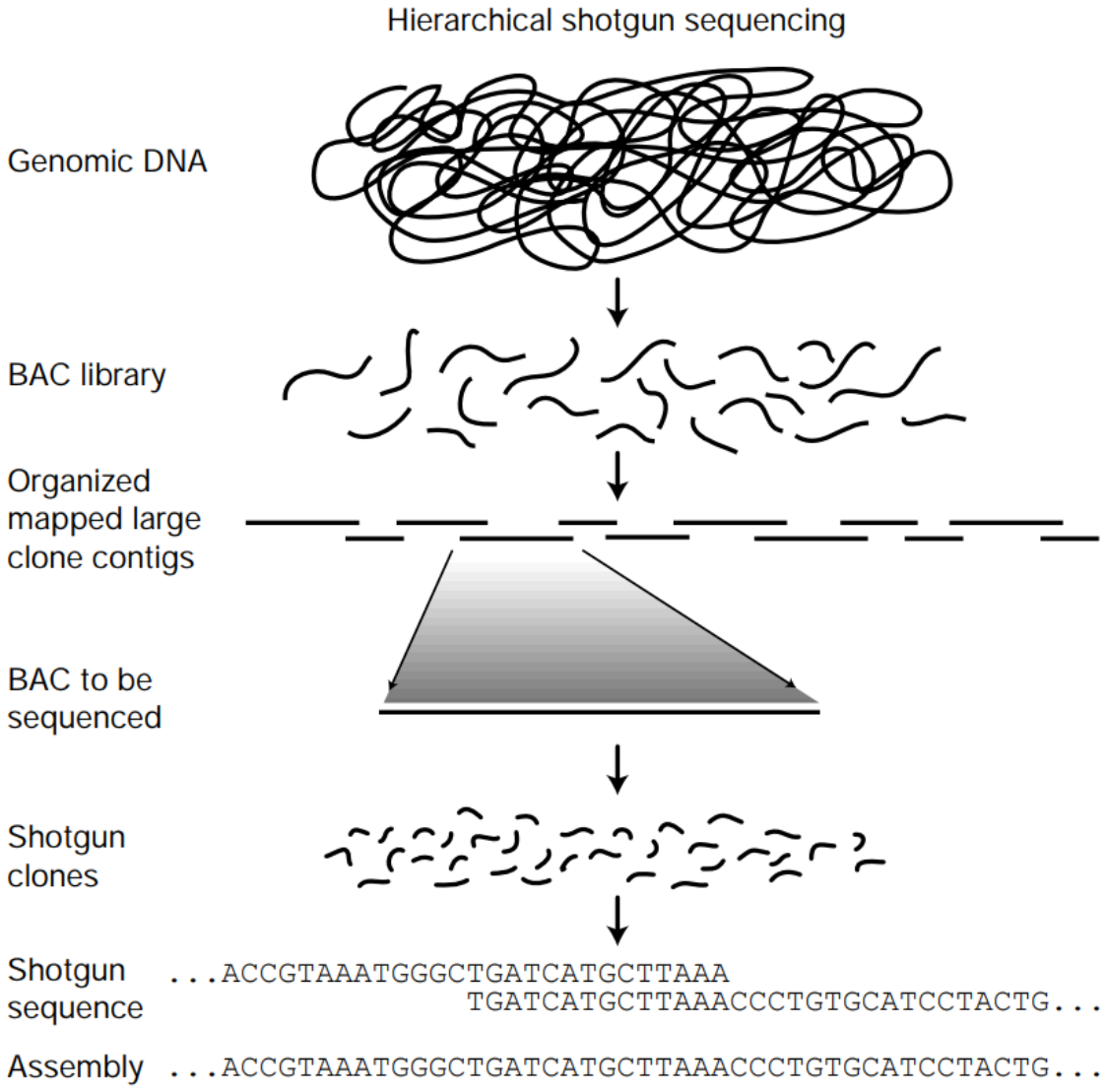
Initial sequencing and analysis of the human genome

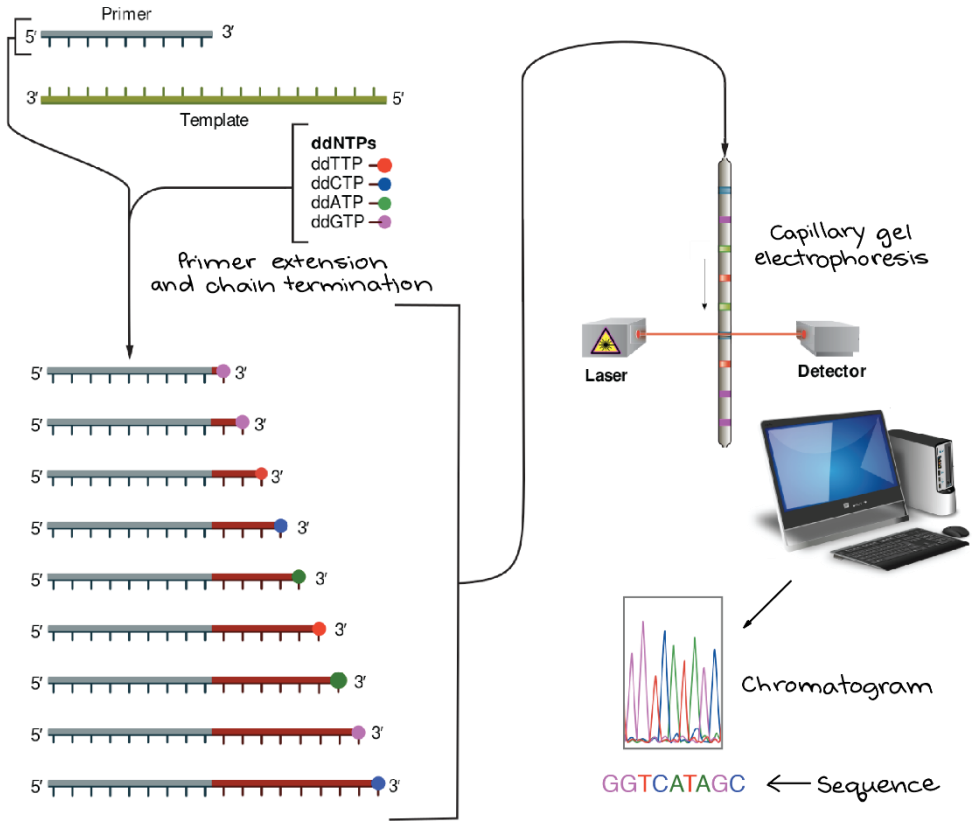
International Human Genome Sequencing Consortium*

** A partial list of authors appears on the opposite page. Affiliations are listed at the end of the paper.*

The human genome holds an extraordinary trove of information about human development, physiology, medicine and evolution. Here we report the results of an international collaboration to produce and make freely available a draft sequence of the human genome. We also present an initial analysis of the data, describing some of the insights that can be gleaned from the sequence.

How did the HGP worked?





Source: International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature. 2001 Feb 15;409(6822):860-921.

To face the increasing need of sequences, technology faced a great development with creation of new sequencing machines and approaches that could guarantee higher output in terms of gigabases of output and lower cost.

This new sequences techniques are referred to as Next Generation Sequencing (Second or Third Generation Sequencers).

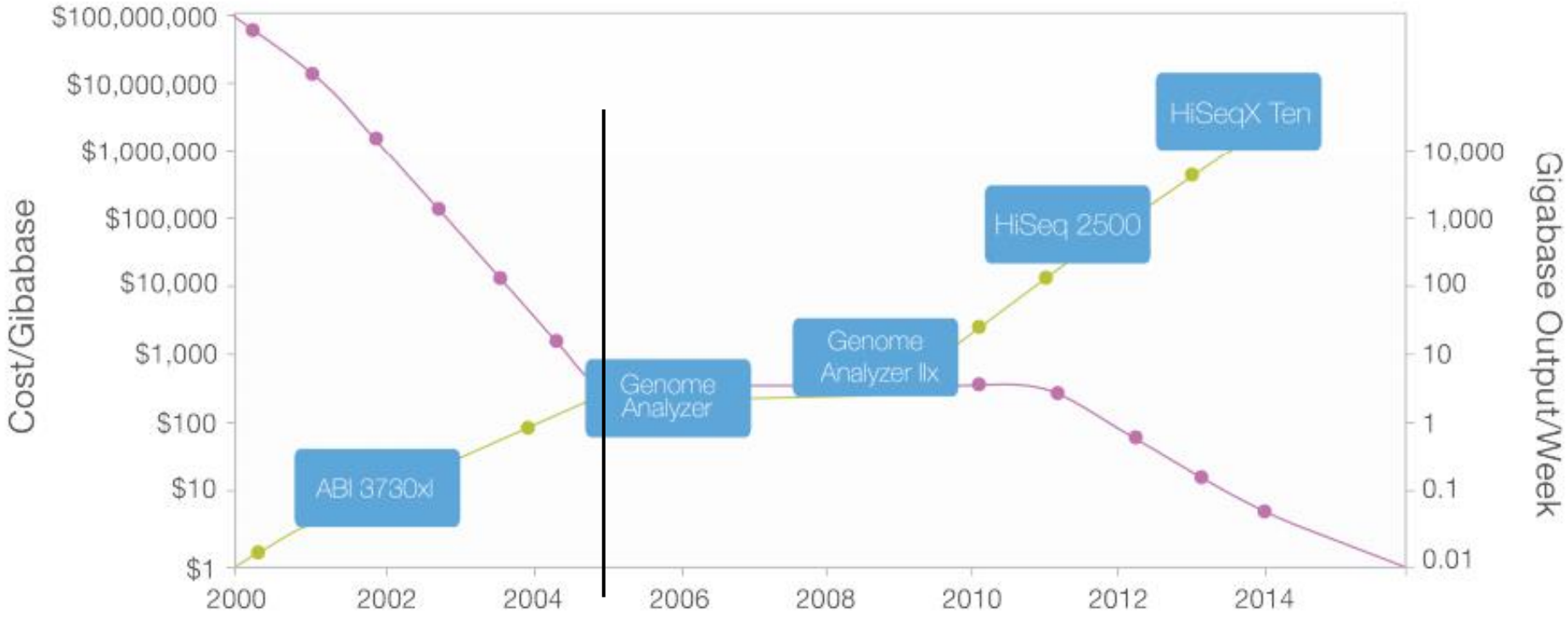
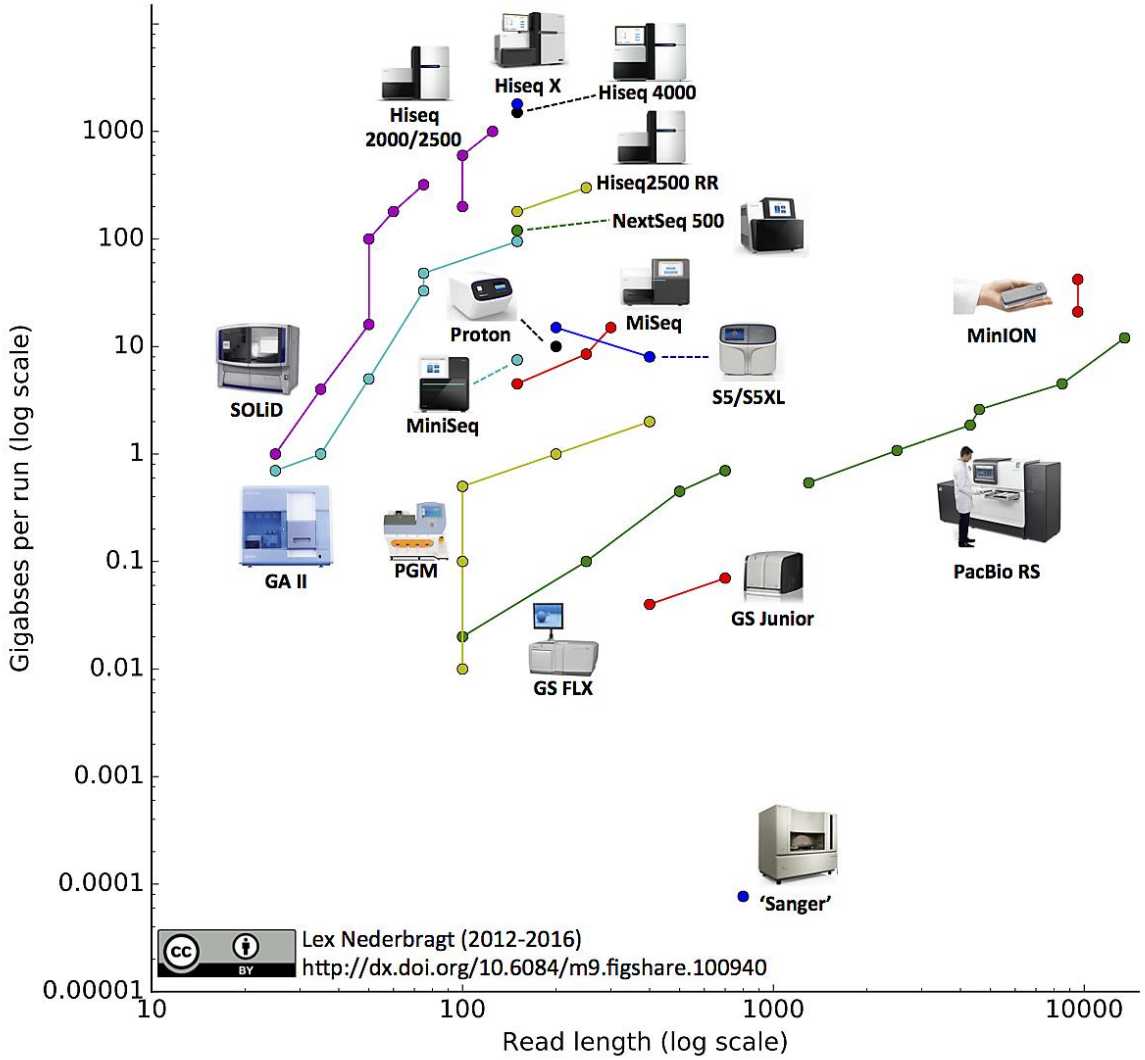
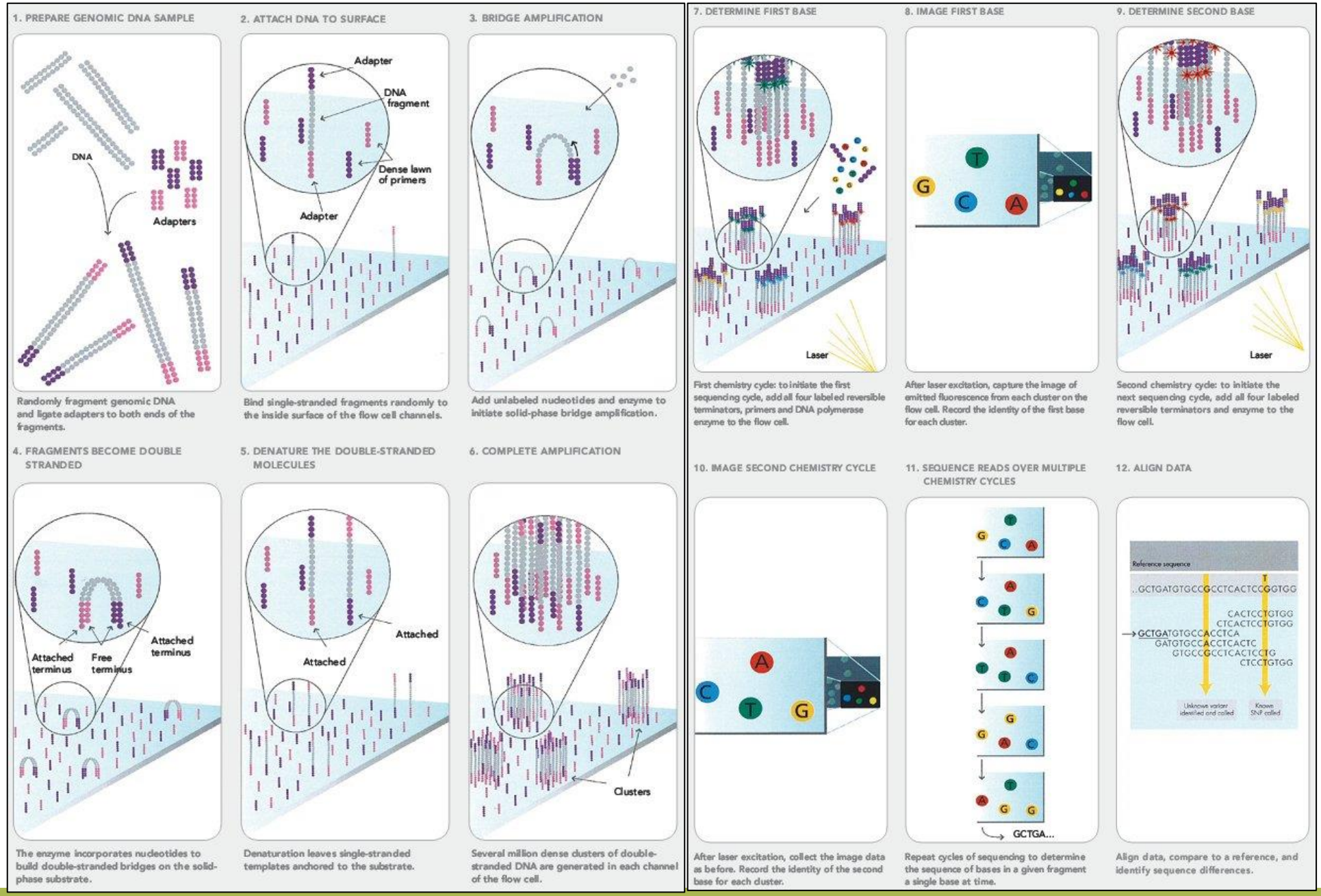
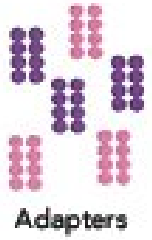


Figure 1: Sequencing Cost and Data Output Since 2000—The dramatic rise of data output and concurrent falling cost of sequencing since 2000. The Y-axes on both sides of the graph are logarithmic.


Hiseq X
Hiseq 4000
Hiseq 2000/2500
Hiseq2500 RR
NextSeq 500
Proton
MiSeq
MinION
SOLiD
MiniSeq
S5/S5XL
GA II
PGM
PacBio RS
GS Junior
GS FLX
'Sanger'
Lex Nederbragt (2012-2016)
http://dx.doi.org/10.6084/m9.figshare.100940
Gigabses per run (log scale)
Read length (log scale)
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
10
100
1000
10000


1. PREPARE GENOMIC DNA SAMPLE
DNA
Adapters
Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.
2. ATTACH DNA TO SURFACE
Adapter
DNA fragment
Dense lawn of primers
Adapter
Bind single-stranded fragments randomly to the inside surface of the flow cell channels.
3. BRIDGE AMPLIFICATION
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.
4. FRAGMENTS BECOME DOUBLE STRANDED
Attached terminus
Free terminus
Attached terminus
The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.
5. DENATURE THE DOUBLE-STRANDED MOLECULES
Attached
Attached
Denaturation leaves single-stranded templates anchored to the substrate.
6. COMPLETE AMPLIFICATION
Clusters
Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.
7. DETERMINE FIRST BASE
Laser
First chemistry cycle: to initiate the first sequencing cycle, add all four labeled reversible terminators, primers and DNA polymerase enzyme to the flow cell.
8. IMAGE FIRST BASE
After laser excitation, capture the image of emitted fluorescence from each cluster on the flow cell. Record the identity of the first base for each cluster.
9. DETERMINE SECOND BASE
Laser
Second chemistry cycle: to initiate the next sequencing cycle, add all four labeled reversible terminators and enzyme to the flow cell.
10. IMAGE SECOND CHEMISTRY CYCLE
After laser excitation, collect the image data as before. Record the identity of the second base for each cluster.
11. SEQUENCE READS OVER MULTIPLE CHEMISTRY CYCLES
GCTGA...
Repeat cycles of sequencing to determine the sequence of bases in a given fragment a single base at time.
12. ALIGN DATA
Reference sequence
..GCTGATGTGCCGCCTCACTCCGGTGG
CACTCCTGTGG
CTCACTCCTGTGG
GCTGATGTGCCACCTCA
GATGTGCCACCTCACTC
GTGCCGCCTCACTCCTG
CTCCTGTGG
Unknown variant identified and called
Known SNP called
Align data, compare to a reference, and identify sequence differences.



Nextera Index Kit – PCR Primers

Index 1 Read

5′ CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

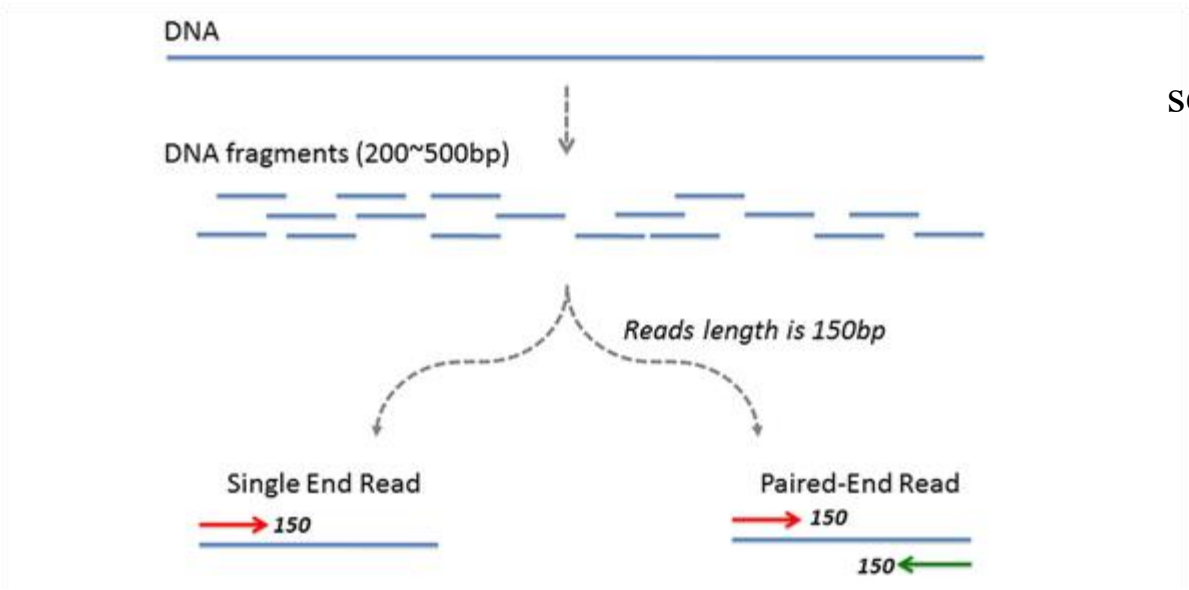
Index 2 Read

5′ AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

Nextera Index Kit - Index 1 (i7) Adapters

Bases in Adapter	i7 Index Name
TCGCCTTA	N701
CTAGTACG	N702
TTCTGCCT	N703
GCTCAGGA	N704
AGGAGTCC	N705
CATGCCTA	N706
GTAGAGAG	N707
CCTCTCTG	N708
AGCGTAGC	N709
CAGCCTCG	N710
TGCCTCTT	N711
TCCTCTAC	N712

Costo/campione con 50,000 sequenze: 50 euro



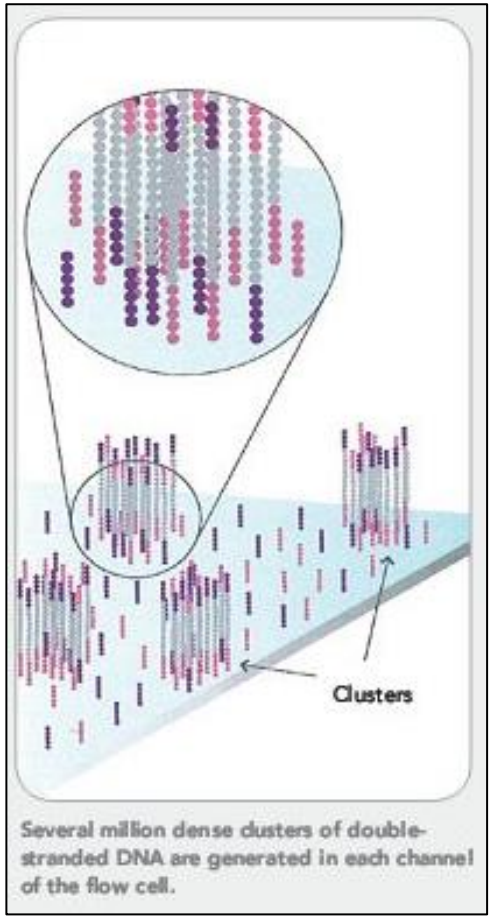
One of the many advantage of NGS, is that some sequencers allow to read the sequence in both directions.

Single-Read Sequencing

Single-read sequencing involves sequencing DNA from only one end, and is the simplest way to utilize Illumina sequencing. By leveraging proprietary reversible terminator chemistry and a novel polymerase, this solution delivers large volumes of high-quality data, rapidly and economically.

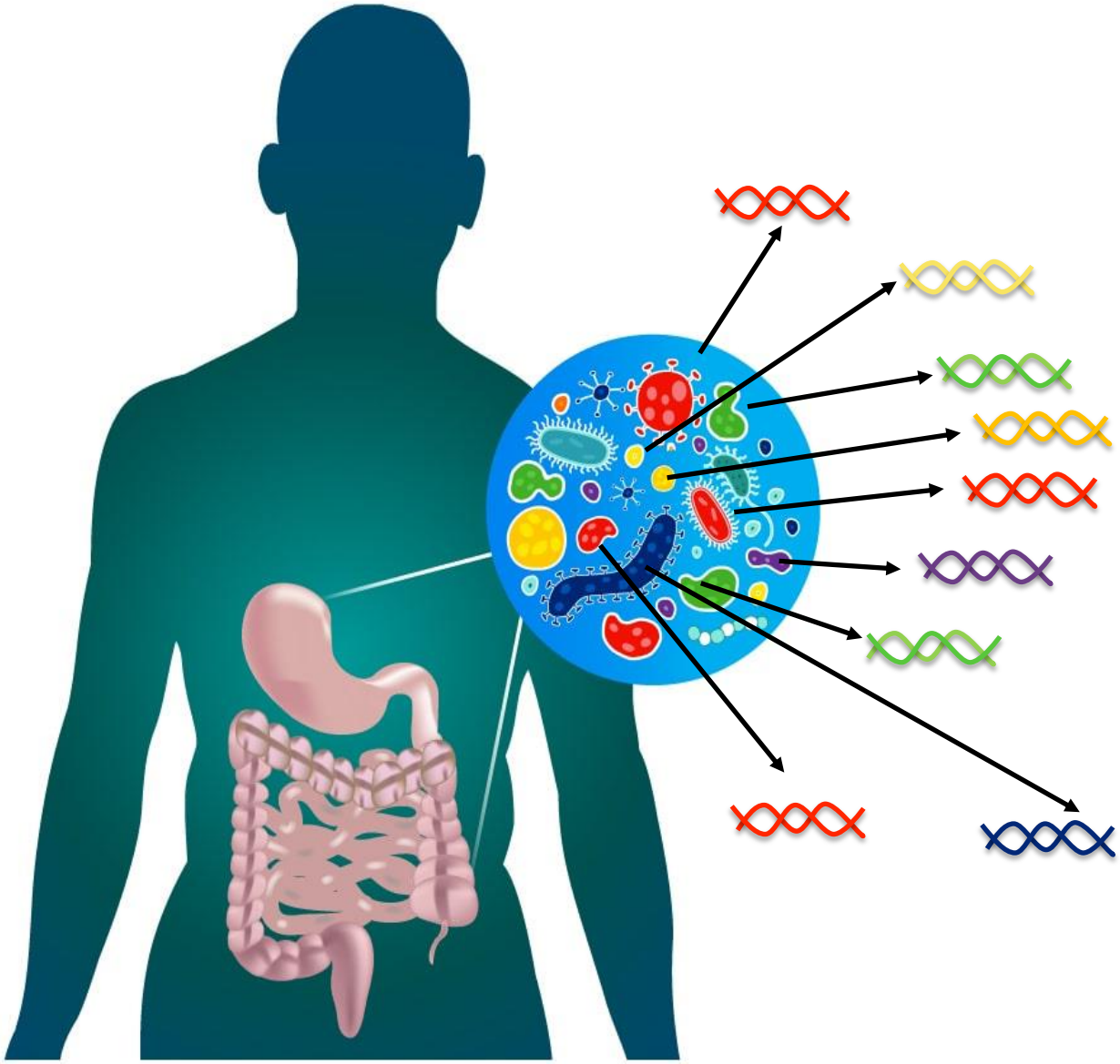
Paired-End DNA Sequencing

Paired-end DNA sequencing reads provide superior alignment across DNA regions containing repetitive sequences, and produce longer contigs for de novo sequencing by filling gaps in the consensus sequence. Paired-end DNA sequencing also detects rearrangements such as insertions, deletions, and inversions.



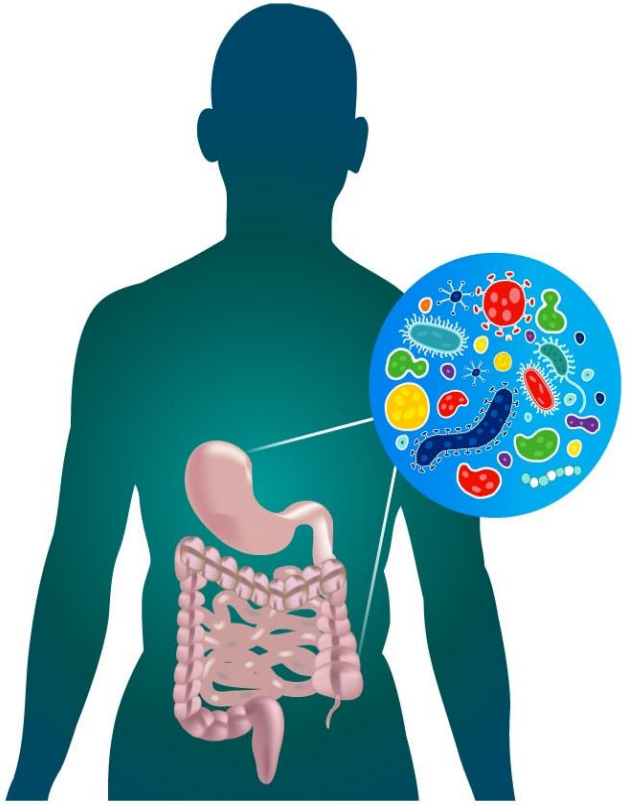
Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

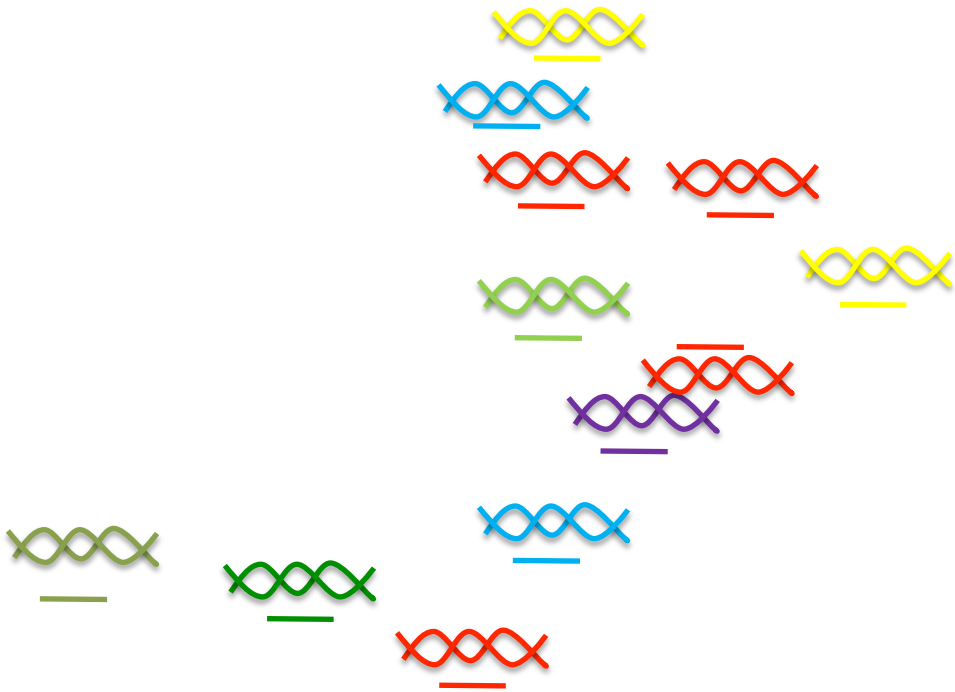
From the development of NGS methods: culture-independent approaches



We could use this to by-pass the culturing step and move directly to investigating the microbiome…

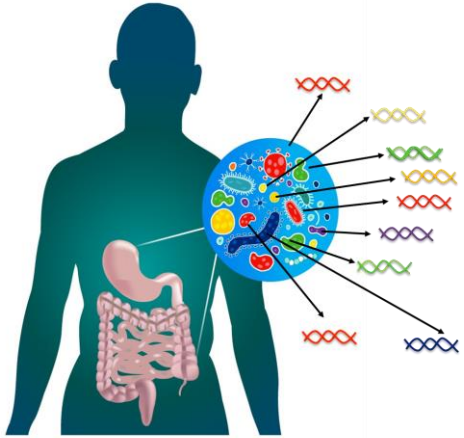
Molecular fingerprinting: culture-independent approach





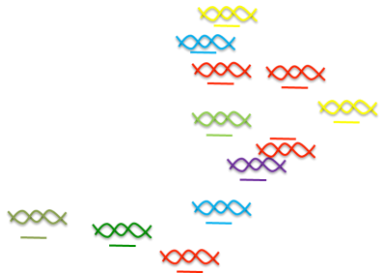
Sequence millions of them

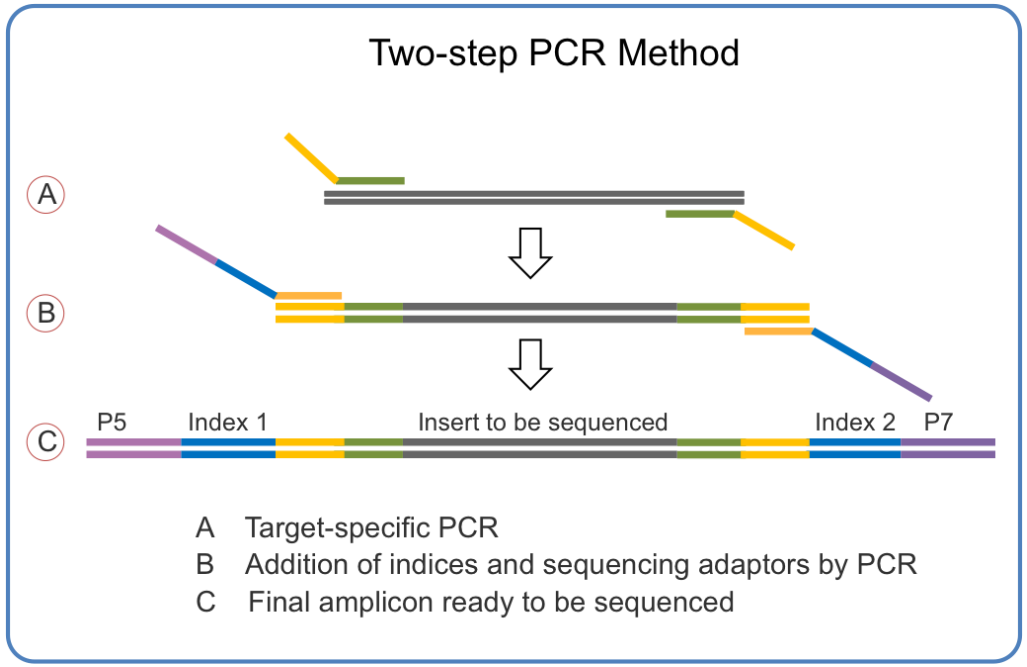
Metabarcoding (or amplicon sequencing)



Amplification of the marker

Library costruction (two-step PCR)



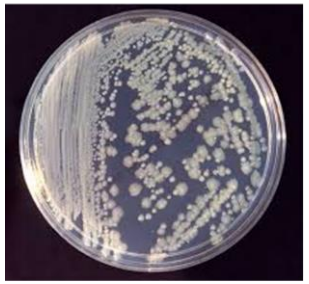


The standard protocol for library preparation is based on amplification of the marker gene, and attachment of "tails" that allow the second PCR.

The second amplification allows the attachment of the adapters for Illumina sequencing (containing also the barcode that allows multiplexing).

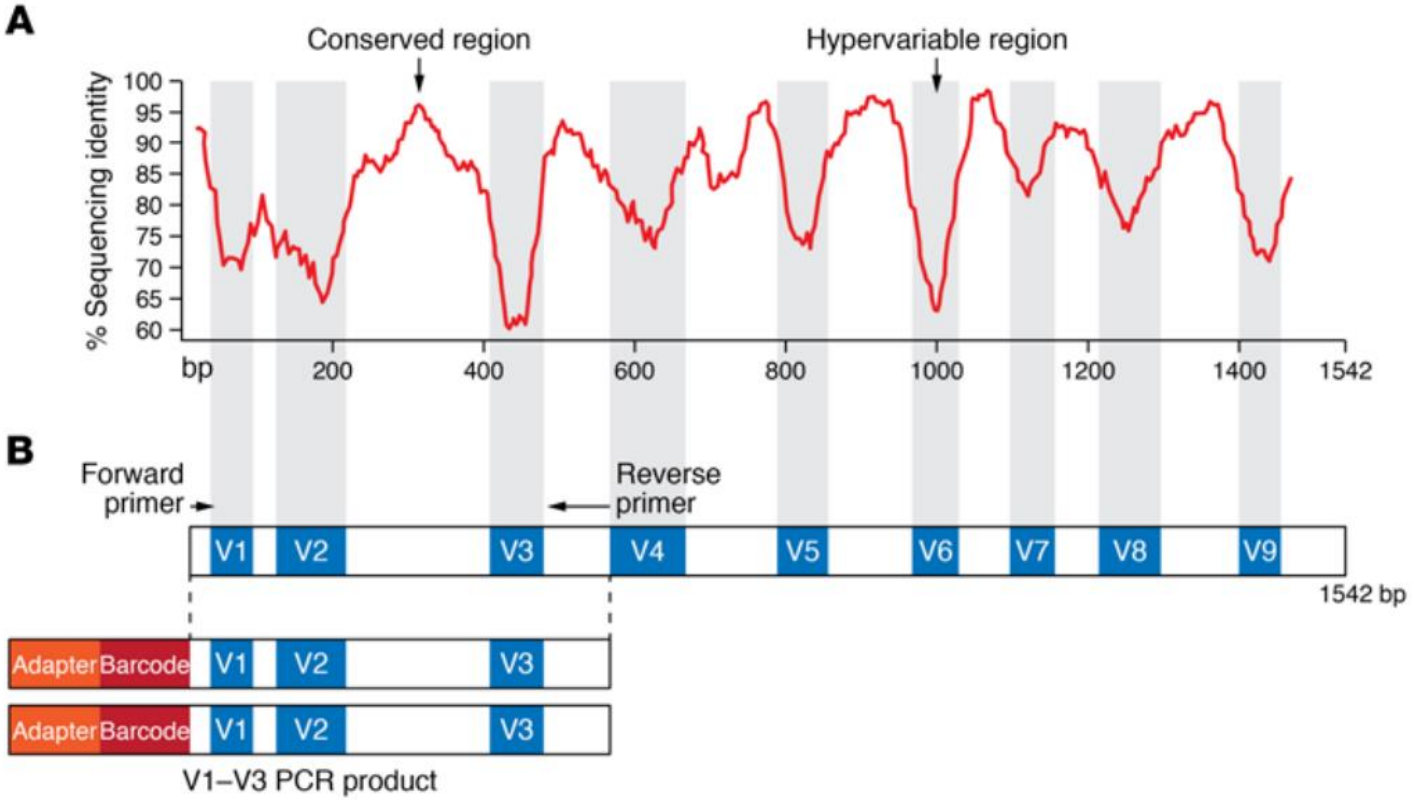
Most commonly used marker

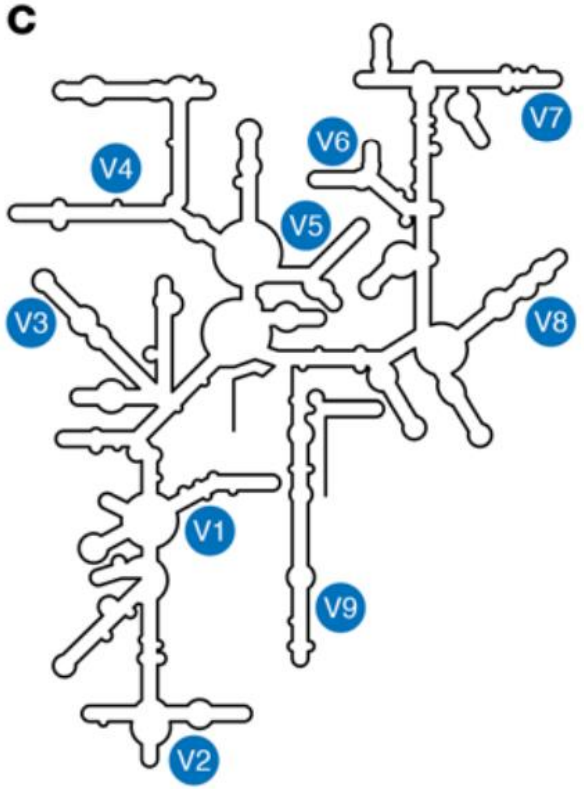
16S to identify bacteria



16S rRNA gene

- Since 1977 for phylogenetics in bacteria (Woese and Fox, 1977)
- Specific hypervariable regions have different discriminations powers for different taxa





Results found in 28 databases

REFSEQ TARGETED LOCI PROJECT

16S ribosomal RNA

16S rRNA is a component of the small subunit of a prokaryotic ribosome. The genes coding for it are used in reconstructing phylogenies, due to ...

Literature	
Bookshelf	492
MeSH	4
NLM Catalog	27
PubMed	91,421
PubMed Central	139,793

Genes	
Gene	63,353
GEO DataSets	2,762
GEO Profiles	953
HomoloGene	1
PopSet	46,431

Proteins	
Conserved Domains	97
Identical Protein Groups	729,892
Protein	370,881,817
Protein Family Models	330
Structure	763

Genomes	
Assembly	0
BioCollections	0
BioProject	12,335
BioSample	283,957
Genome	23
Nucleotide	46,465,622
SRA	1,647,100
Taxonomy	0

Clinical	
ClinicalTrials.gov	889
ClinVar	8
dbGaP	27
dbSNP	0
dbVar	0
GTR	14
MedGen	3
OMIM	23

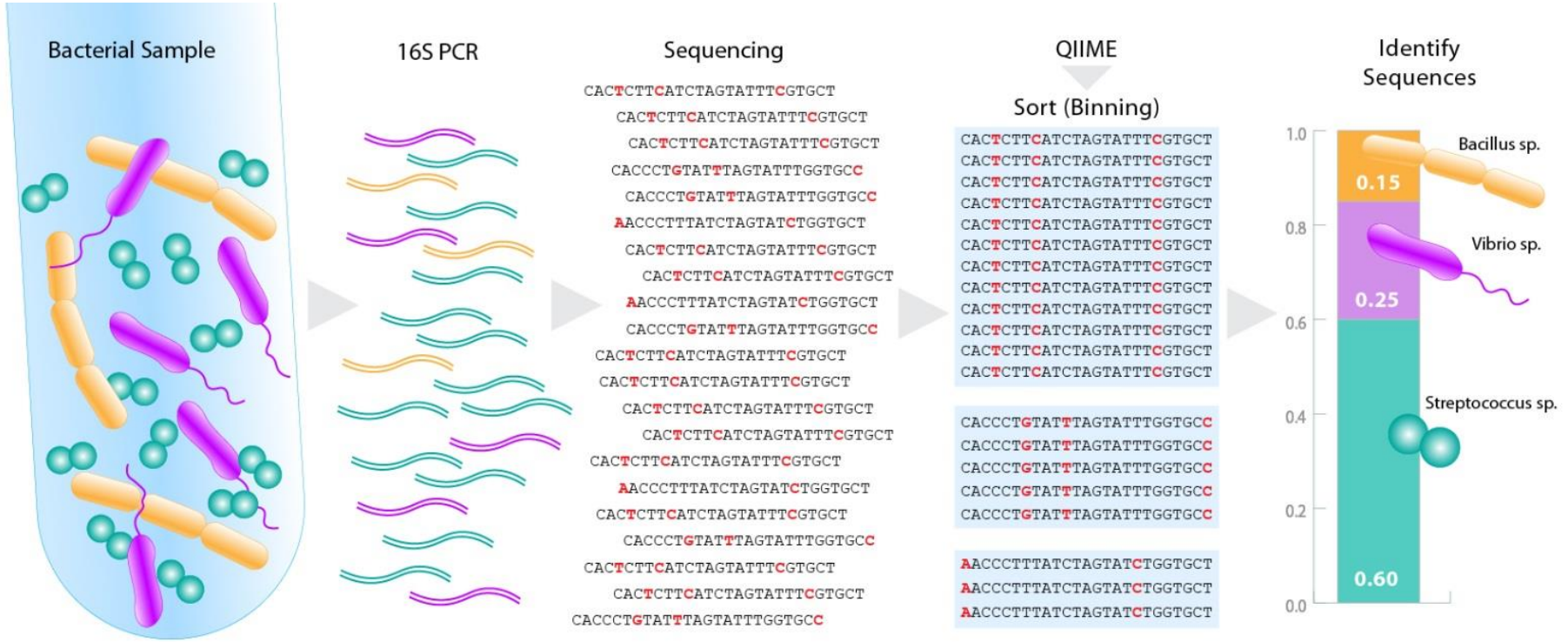
PubChem	
BioAssays	898
Compounds	0
Pathways	0
Substances	54

There are also specialized databases containing 16S sequences such as:

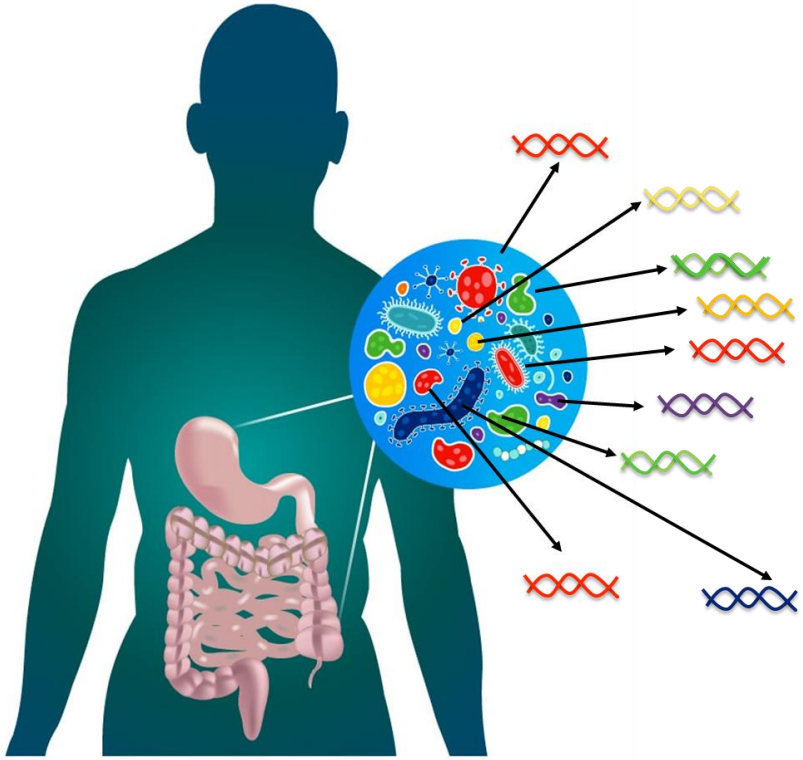
green genes
16S rRNA gene database and workbench compatible with ARB
greengenes.lbl.gov

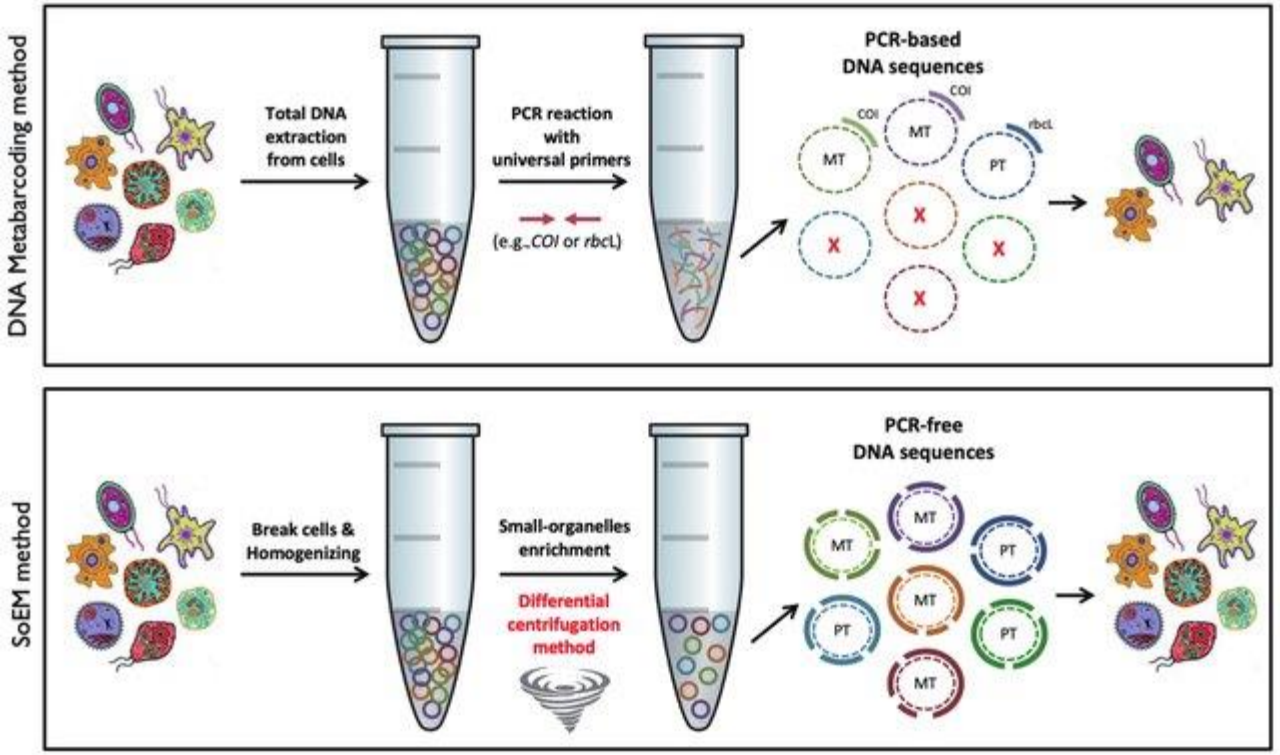
silva
high quality ribosomal RNA databases

From samples to sequence to taxonomy: how the bioinformatic tools work?



NGS to the rescue: metabarcoding vs metagenomics





Metagenomics

DNA extraction

Sequencing

Reads

100-150 bp

Assembly

Contigs

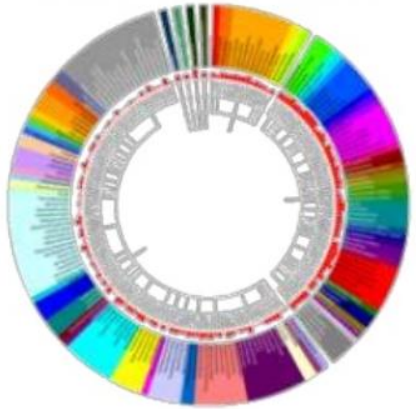
1000+ bp

Search against database

Phylogenetic classification

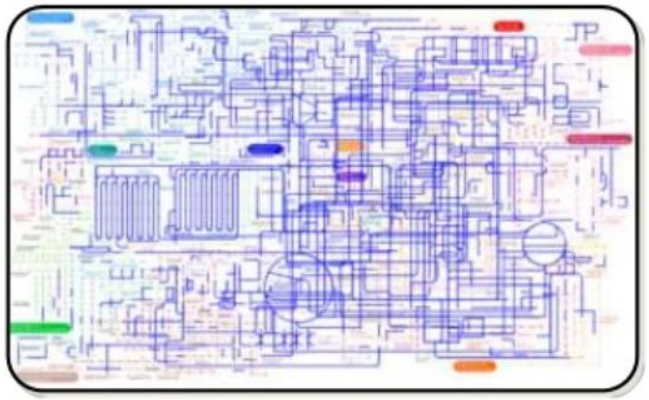
Who is there?

Bacterium A
Bacterium B
...
Bacterium X



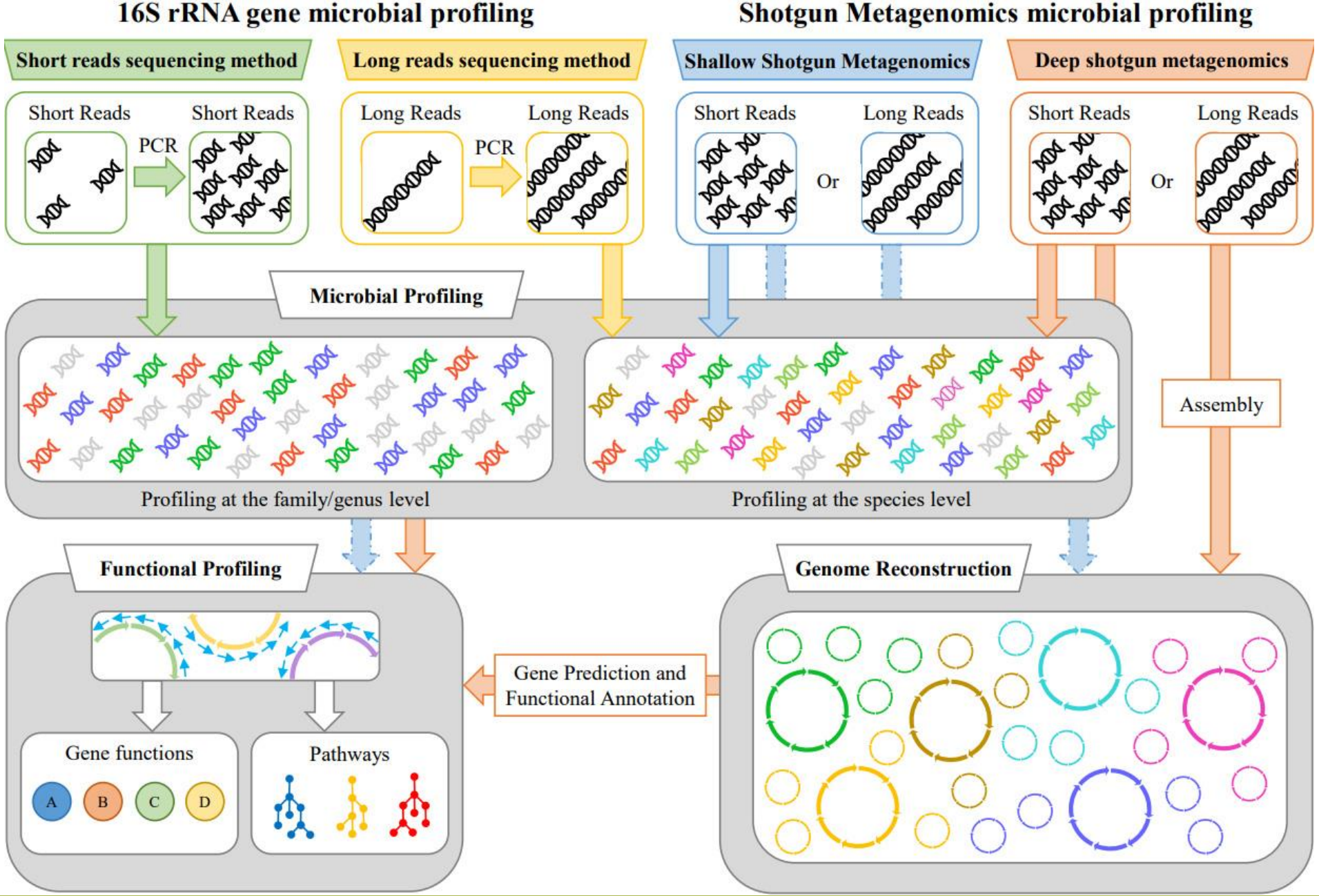
Functional classification

What can they do?

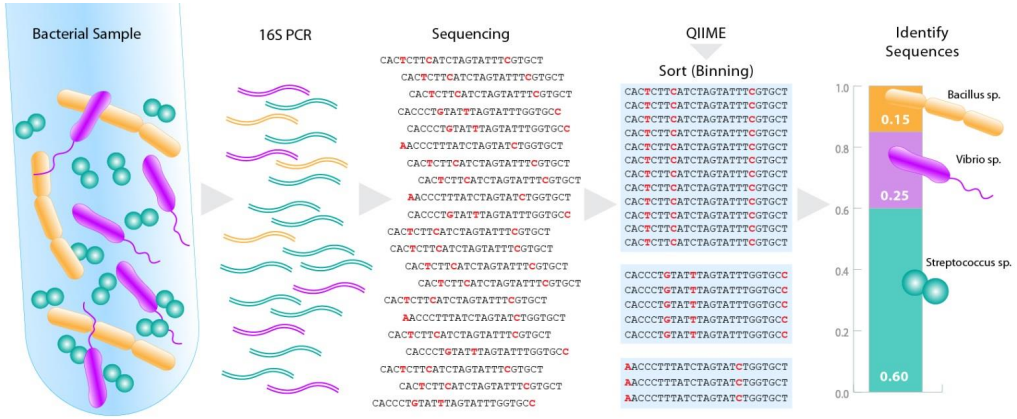


Gene A
Gene B
...
Gene X

Summary


16S rRNA gene microbial profiling
Shotgun Metagenomics microbial profiling
Short reads sequencing method
Long reads sequencing method
Shallow Shotgun Metagenomics
Deep shotgun metagenomics
Short Reads
PCR
Short Reads
Long Reads
PCR
Long Reads
Short Reads
Or
Long Reads
Short Reads
Or
Long Reads
Microbial Profiling
Profiling at the family/genus level
Profiling at the species level
Assembly
Functional Profiling
Genome Reconstruction
Gene Prediction and
Functional Annotation
Gene functions
A
B
C
D
Pathways

From samples to sequence to taxonomy: how the bioinformatic tools work?



ASV= AMPLICON SEQUENCE VARIANT

For shotgun:

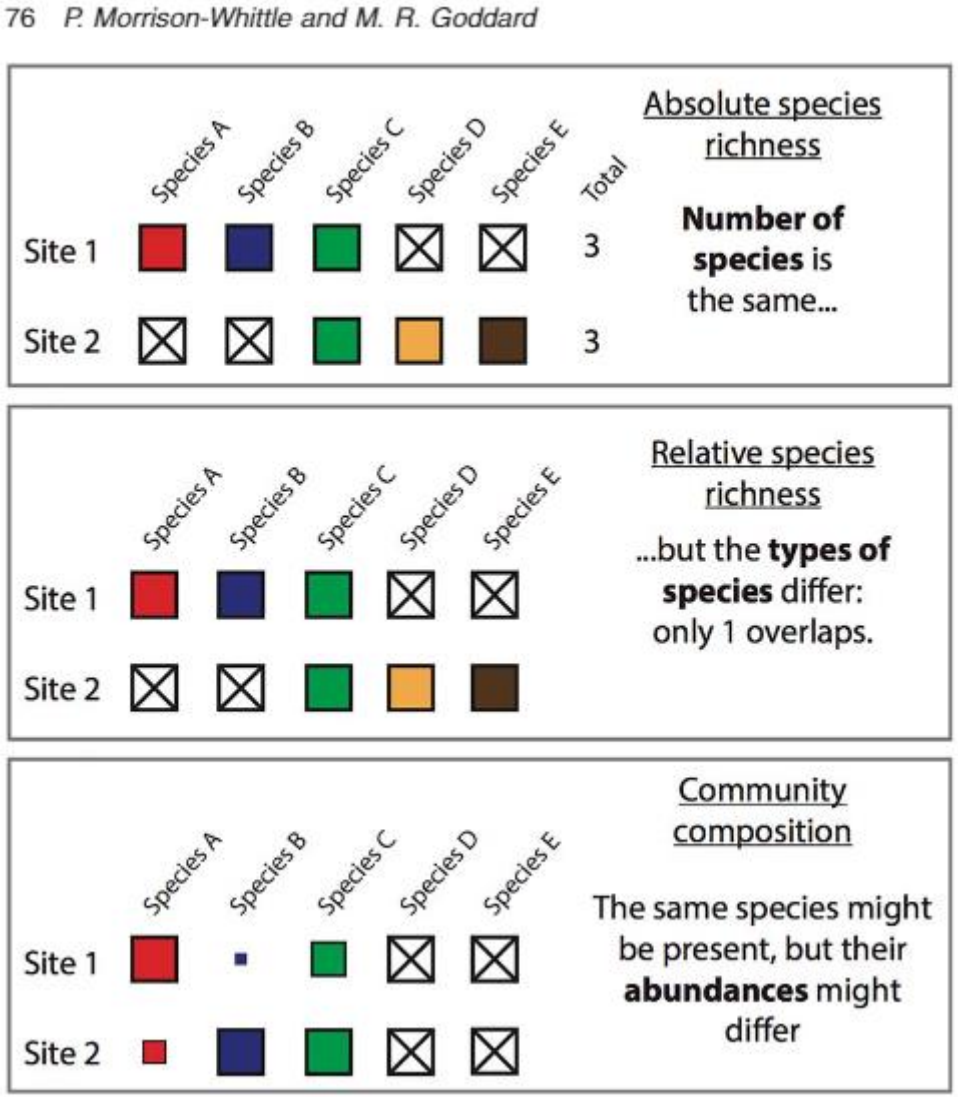
- we skip the amplification step
- We will have 2 main output tables
  - One about the bacterial composition
  - One with functional profile of the community.

	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9
ASV1	66	178	7	7	1360	1335	1292	395	377
ASV2	94	105	10	12	1078	664	174	105	33
ASV3	887	0	598	575	56	491	670	796	0
ASV4	188	0	66	33	0	572	482	1009	0
ASV5	366	0	156	0	0	0	0	0	0
ASV6	287	0	135	0	0	0	0	0	0
ASV7	462	0	3	10	0	0	0	0	0
ASV8	0	323	0	0	124	0	0	0	0
ASV9	4	0	0	0	0	0	14	820	0
ASV10	0	0	0	189	0	727	0	1001	0
ASV11	0	0	0	0	0	0	488	178	0

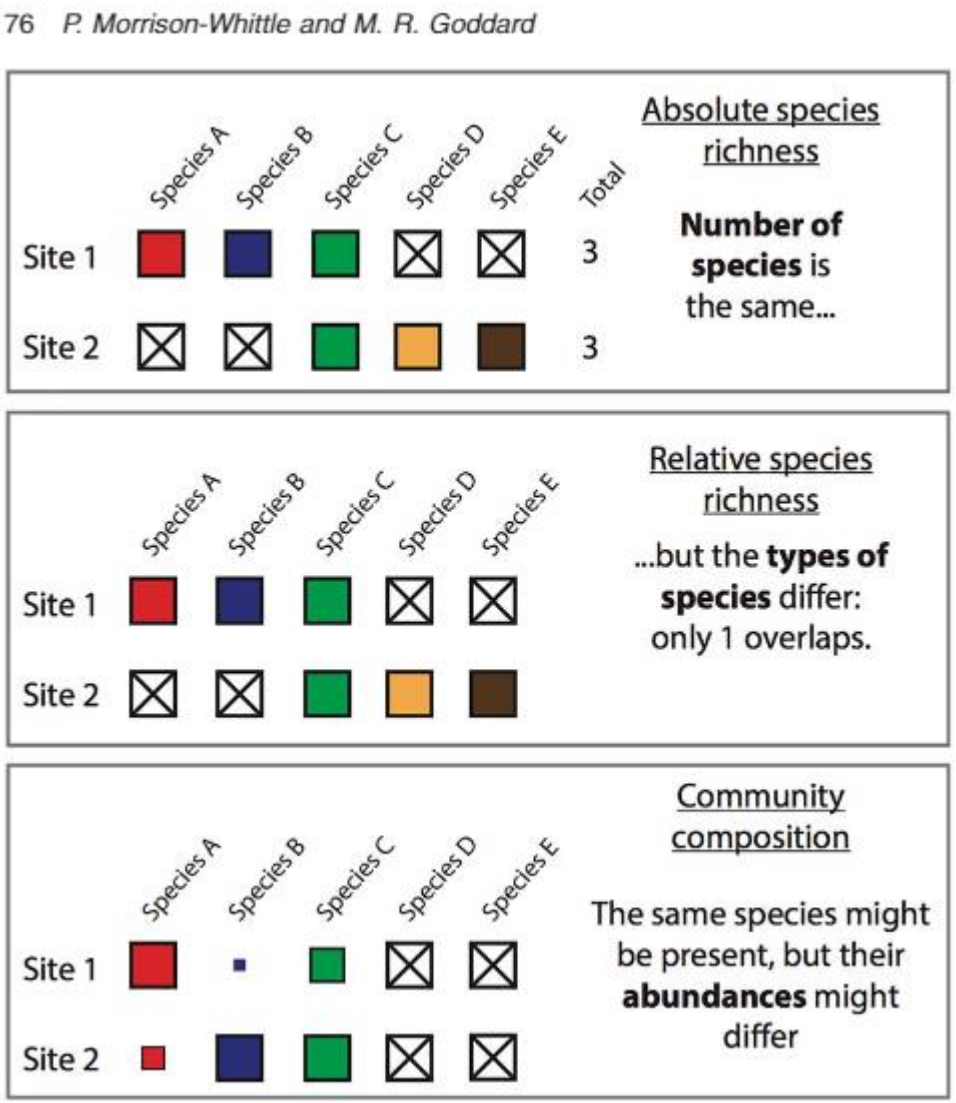
Comparing the samples

There are three commonly used measures:

- Alpha-diversity
- Beta-diversity
- Gamma-diversity

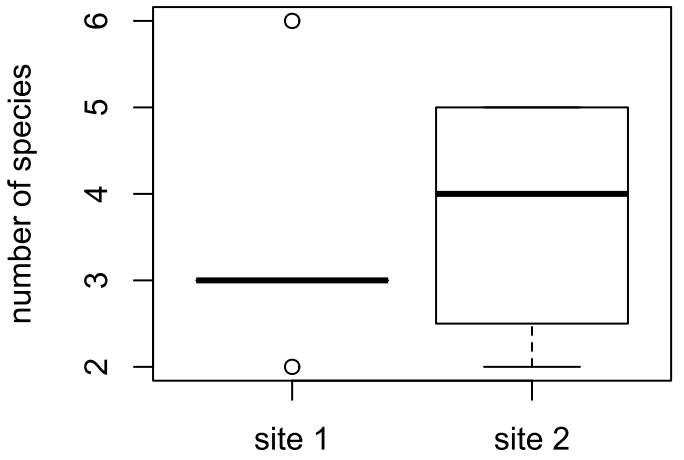


Comparing the samples



	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9
SV1	94	105	10	12	1078	664	174	105	33
SV2	93	11	8	15	2	19	66	63	217
SV3	0	0	10	0	0	0	0	0	0
SV4	0	0	0	0	9	0	0	0	0
SV5	25	0	0	0	0	0	0	0	0
SV6	0	37	0	0	0	0	0	0	0
SV7	0	0	4	0	0	5	0	2	0
SV8	0	0	0	0	0	0	0	0	0
SV9	0	0	0	0	0	8	0	0	0
SV10	0	0	0	0	0	10	0	4	0
SV11	0	0	25	0	0	0	0	0	0
SV12	0	0	3	0	0	0	0	10	10

sampleID	species	type
Sample1	3	site 1
Sample2	3	site 1
Sample3	6	site 1
Sample4	2	site 1
Sample5	3	site 1
Sample6	5	site 2
Sample7	2	site 2
Sample8	5	site 2
Sample9	3	site 2

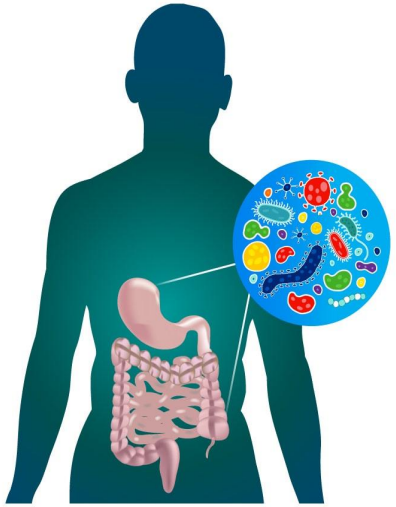


	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9
Sample1	0.00	0.50	0.71	0.33	0.50	0.67	0.33	0.67	0.50
Sample2	0.50	0.00	0.71	0.33	0.50	0.67	0.33	0.67	0.50
Sample3	0.71	0.71	0.00	0.67	0.71	0.67	0.67	0.67	0.71
Sample4	0.33	0.33	0.67	0.00	0.67	0.33	0.33	0.67	0.50
Sample5	0.50	0.50	0.71	0.67	0.00	0.67	0.67	0.67	0.71
Sample6	0.67	0.67	0.67	0.33	0.67	0.00	0.33	0.67	0.50
Sample7	0.33	0.33	0.67	0.33	0.67	0.33	0.00	0.67	0.50
Sample8	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.00	0.71
Sample9	0.50	0.50	0.71	0.50	0.71	0.50	0.50	0.71	0.00

	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9
Sample1	0.00	0.60	0.93	0.87	0.92	0.86	0.45	0.83	0.64
Sample2	0.60	0.00	0.93	0.85	0.91	0.84	0.58	0.83	0.88
Sample3	0.93	0.93	0.00	0.74	0.99	0.96	0.94	0.96	0.93
Sample4	0.87	0.85	0.74	0.00	0.99	0.96	0.89	0.85	0.90
Sample5	0.92	0.91	0.99	0.99	0.00	0.41	0.85	0.91	0.97
Sample6	0.86	0.84	0.96	0.96	0.41	0.00	0.74	0.83	0.94
Sample7	0.45	0.58	0.94	0.89	0.85	0.74	0.00	0.34	0.75
Sample8	0.83	0.83	0.96	0.85	0.91	0.83	0.34	0.00	0.69
Sample9	0.64	0.88	0.93	0.90	0.97	0.94	0.75	0.69	0.00

There are also indexes that could be used to measure species richness (Shannon's, Simpson's and Chao's are the most important ones)

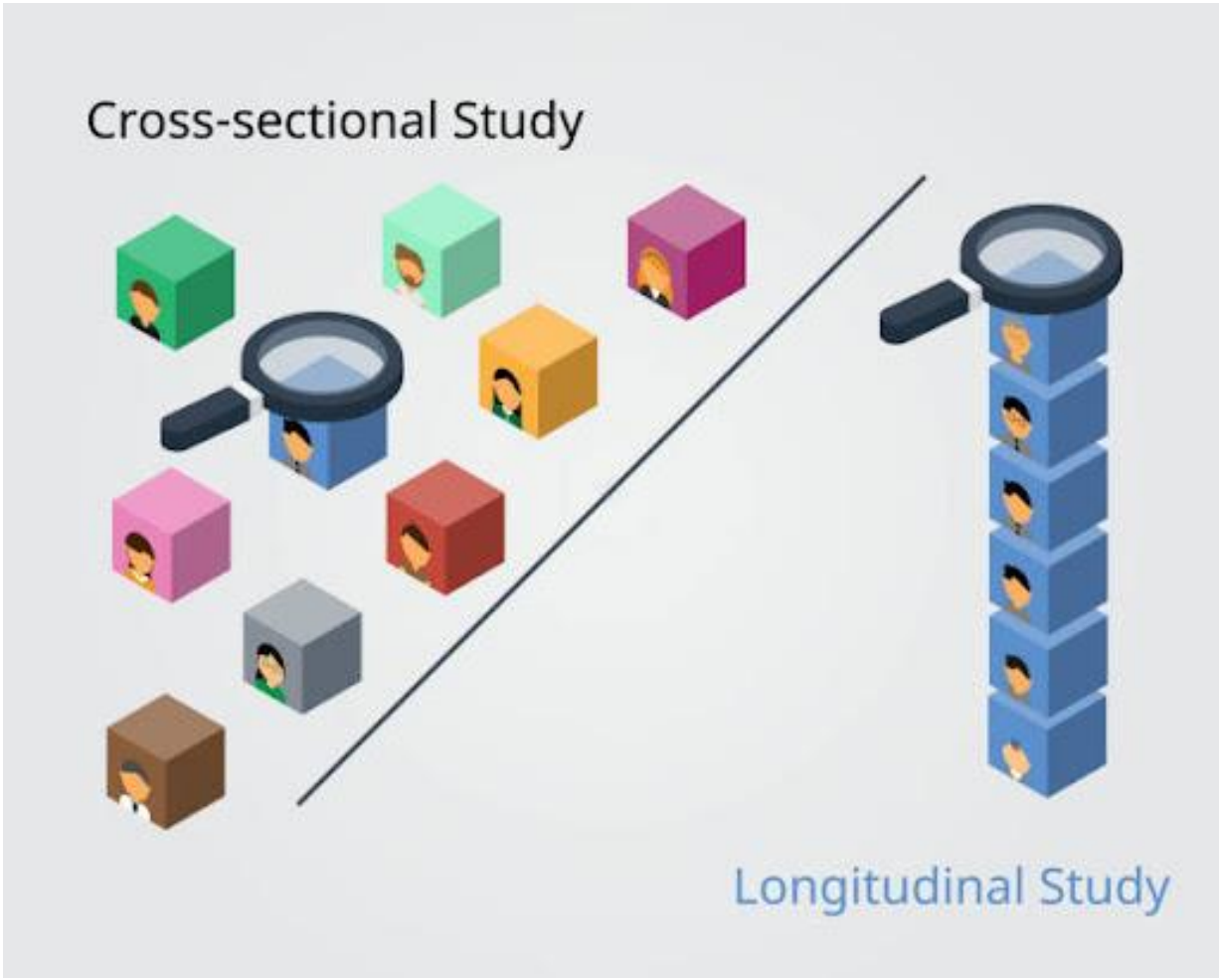
Types of studies to look at the human microbiome



If we can access human samples….

PROS:
In human

CONS:
Recruitment is difficult
Need ethical approval
Cannot share sensitive information
need to protect the privacy of the participants
High inter-individual variability



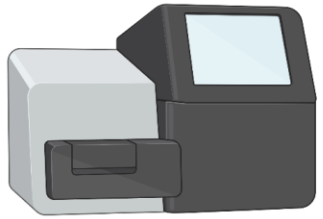
Key considerations in microbiome research

1 Study design

2 Sample collection

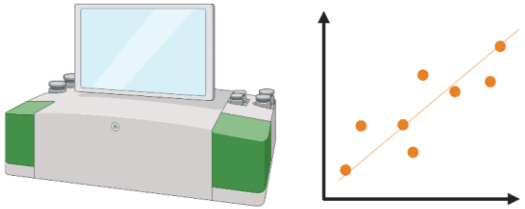
3 Sample processing

4 Sequencing

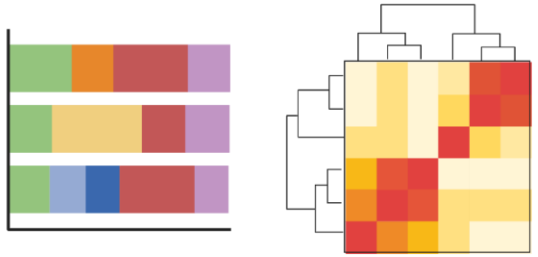


5 Sequence pre-processing

6 Contamination assessment



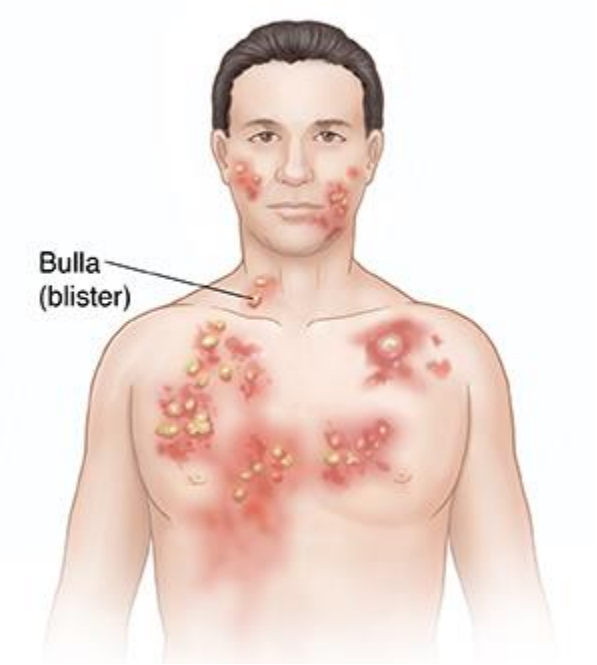
7 Data analysis and visualization



8 Deposit sequence data

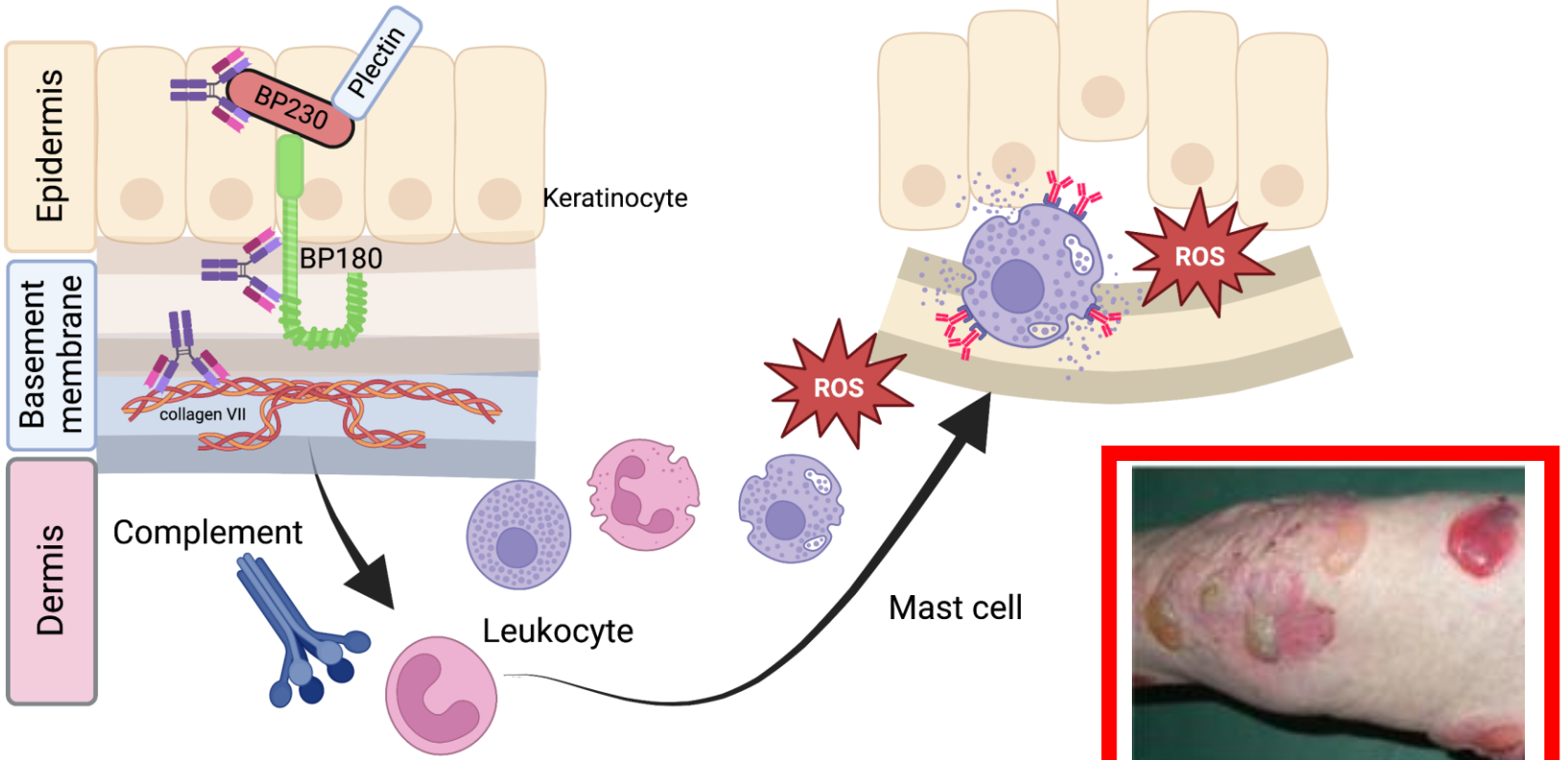
Microbiota in Bullous Pemphigoid:

Is only skin microbiome involved in the pathogenesis of the disease?

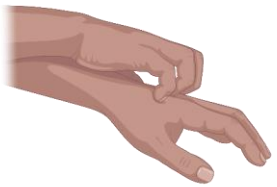


https://mountnittany.org/wellness-article/bullous-pemphigoid-understanding

Bullous pemphigoid is an autoimmune disease.



Elderly



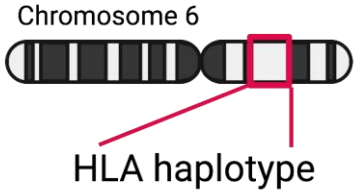
Itchy

Increased death

Sitaru. 2009. *J Invest Derm.*

Factors associated with BP

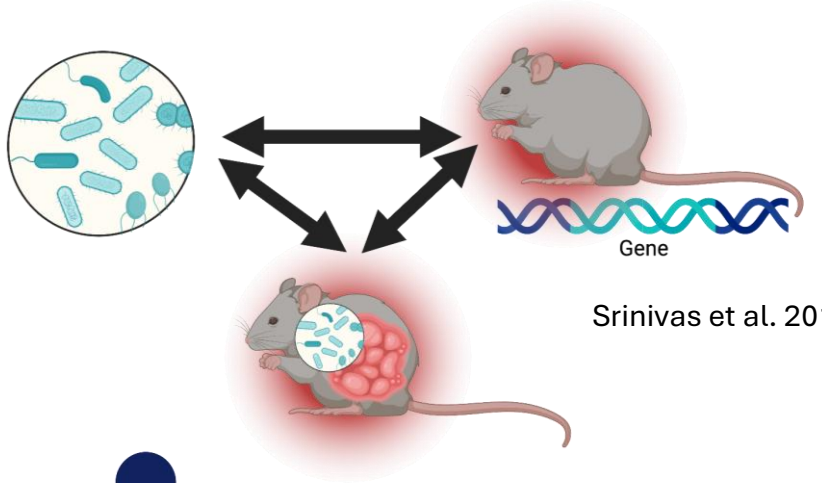
Known factors



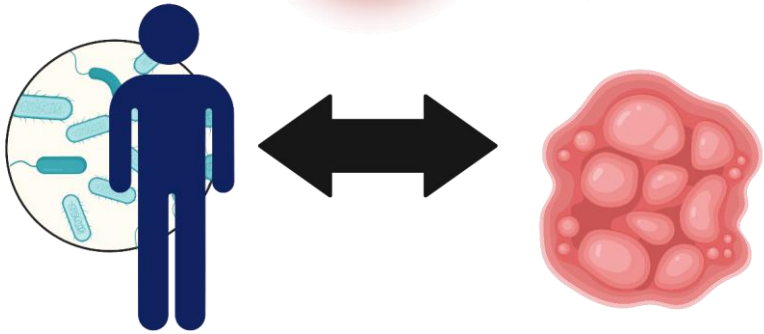
Age

Many medications

Skin microbiota?



Srinivas et al. 2013. *Nat Comm.*



Miodovnik et al. 2017. *Exp Dermatol.*

Candidate taxa & microbial diversity uncharacterized

Journal of Advanced Research 44 (2023) 71–79

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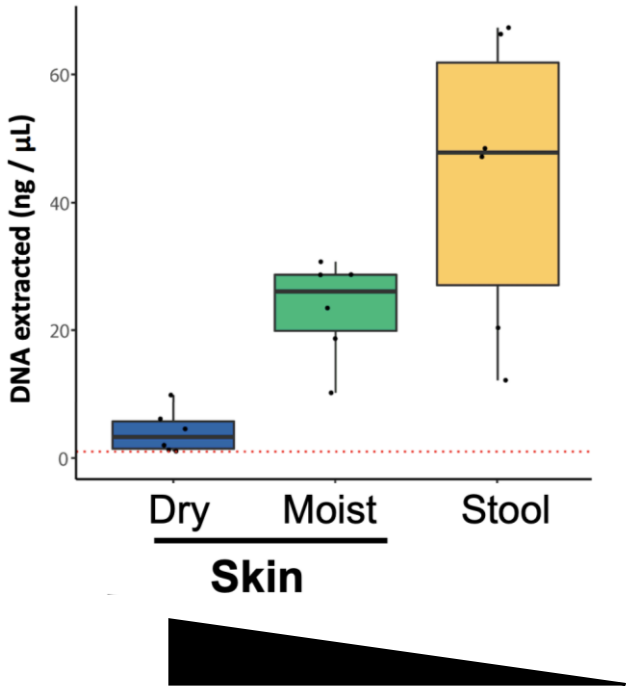
journal homepage: www.elsevier.com/locate/jare

Original Article

Characterization of the skin microbiota in bullous pemphigoid patients and controls reveals novel microbial indicators of disease

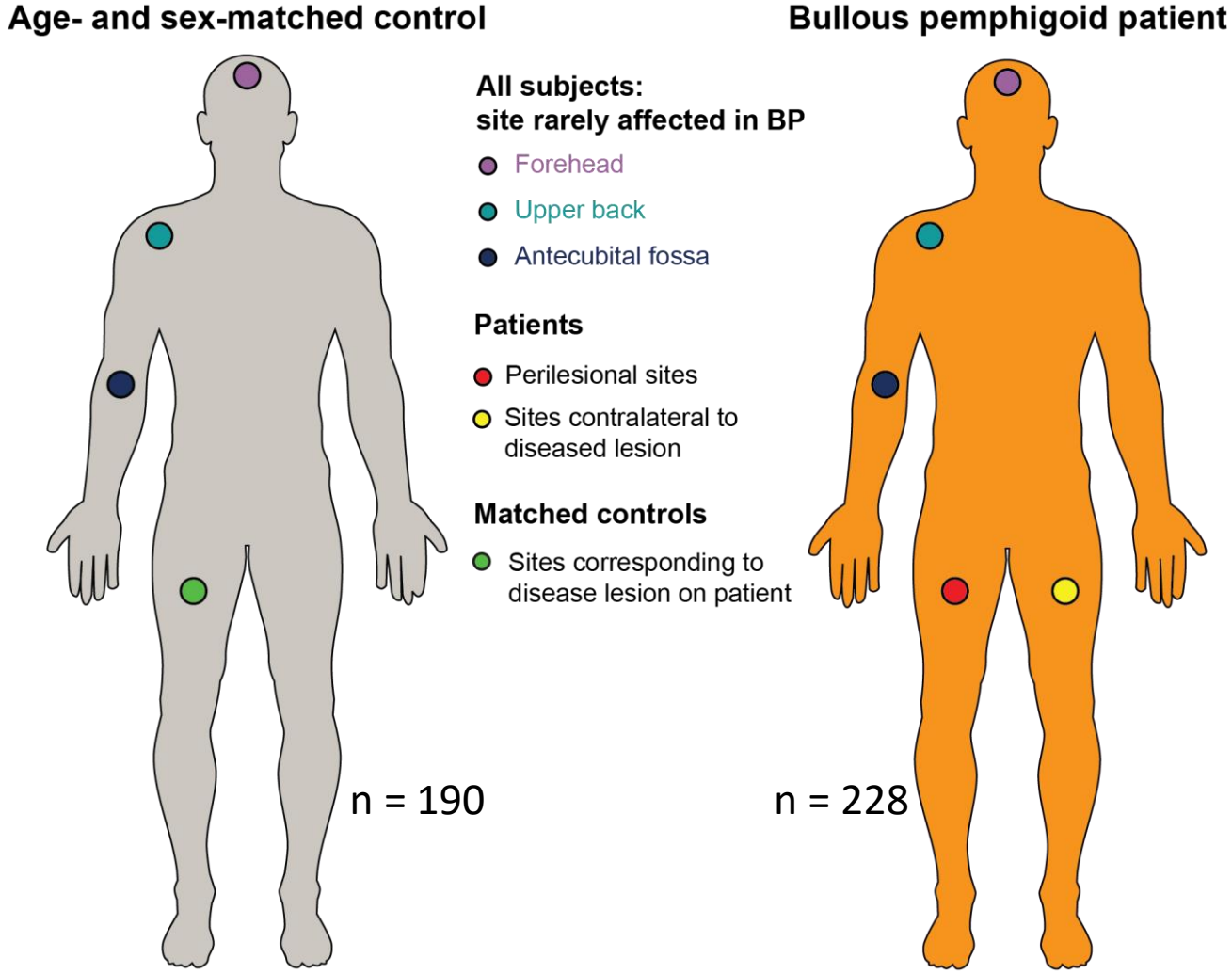
Meriem Belheouane [a,b,c,1], Britt M. Hermes [a,b,1], Nina Van Beek [d], Sandrine Benoit [e], Philippe Bernard [f], Kossara Drenovska [g], Sascha Gerdes [h], Regine Gläser [h], Matthias Goebeler [e], Claudia Günther [i], Anabelle von Georg [d], Christoph M. Hammers [d,r], Maike M. Holtsche [d], Bernhard Homey [j], Orsolya N. Horváth [k], Franziska Hübner [d], Beke Linnemann [d], Pascal Joly [l], Dalma Márton [m], Aikaterini Patsatsi [n], Claudia Pföhler [o], Miklós Sárdy [k,m], Laura Huilaja [p], Snejina Vassileva [g], Detlef Zillikens [d,q], Saleh Ibrahim [q,r,s], Christian D. Sadik [d,q], Enno Schmidt [d,q,r,1], John F. Baines [a,b,1,*]

n = 2956

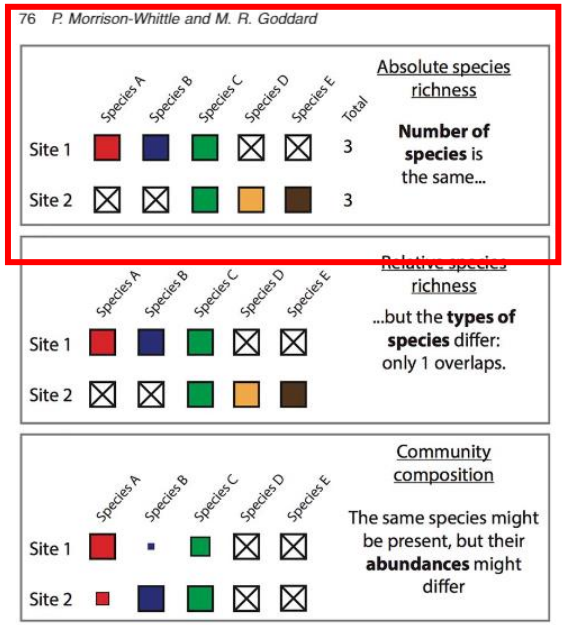


Contamination risk

Skin Microbial Sampling Overview

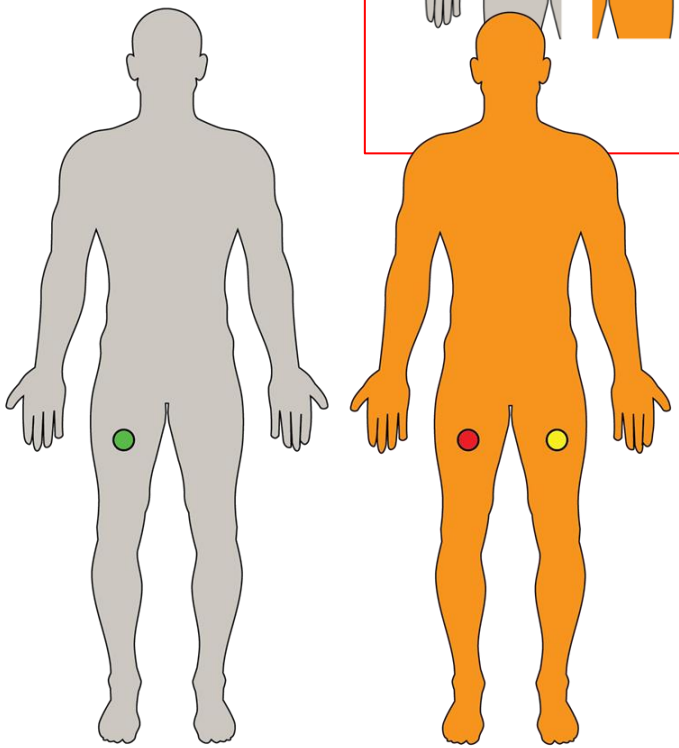


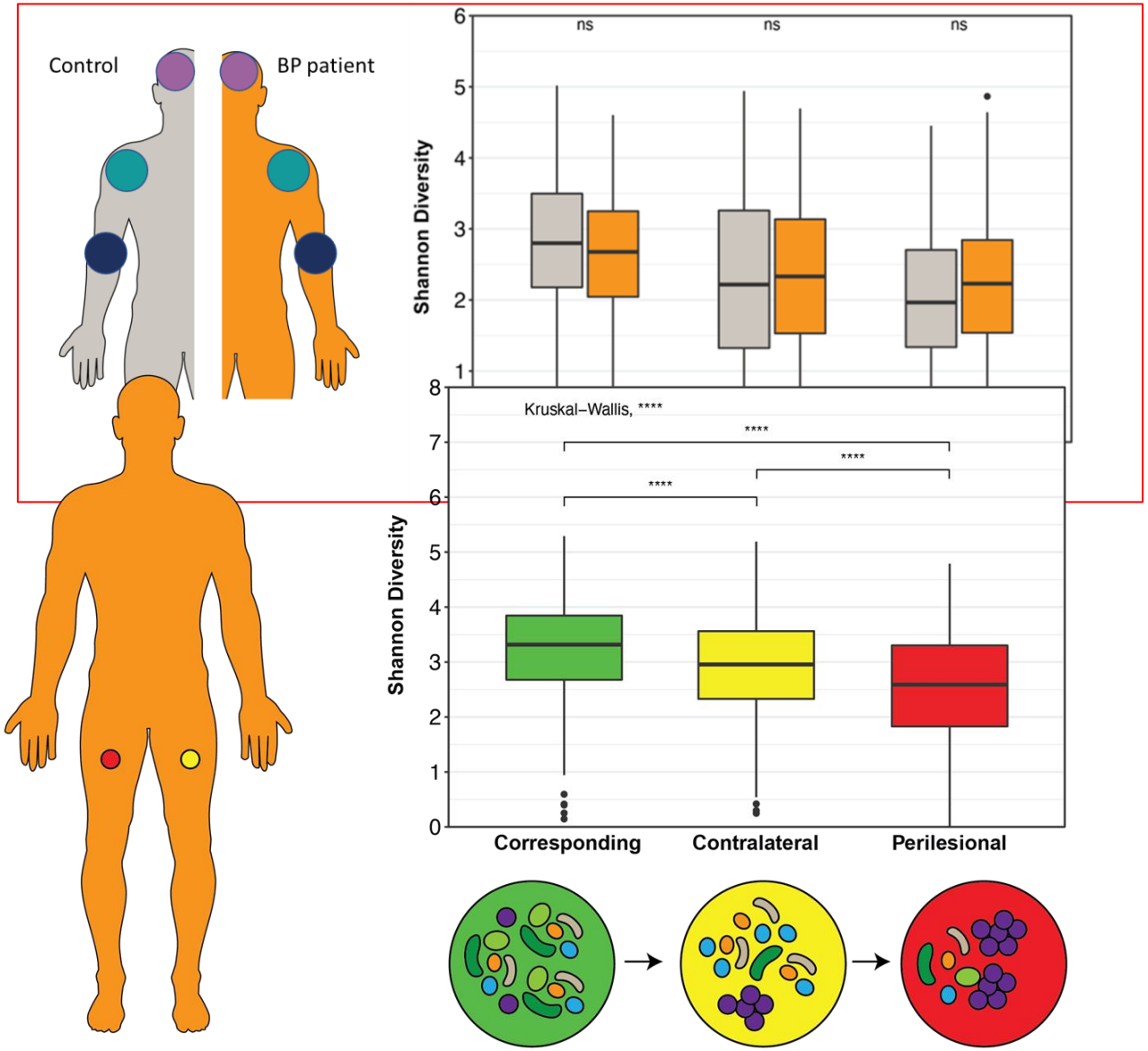
Thanks to Britt Hermes for the amazing figures

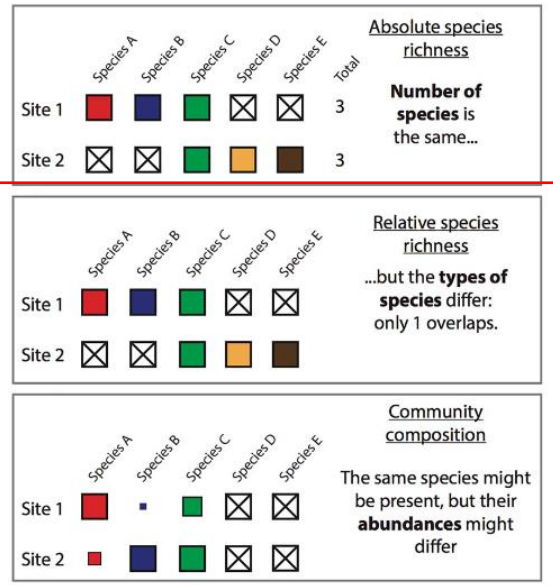


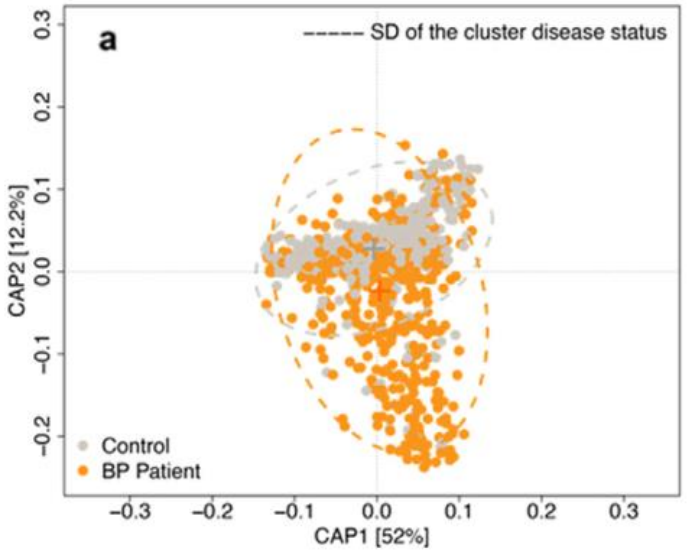
In the sites that are rarely affected by BP…

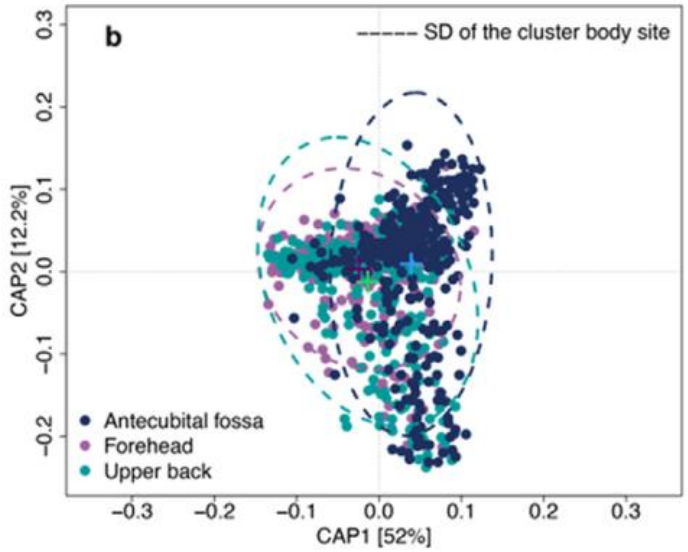
In the sites that are usually affected by BP…

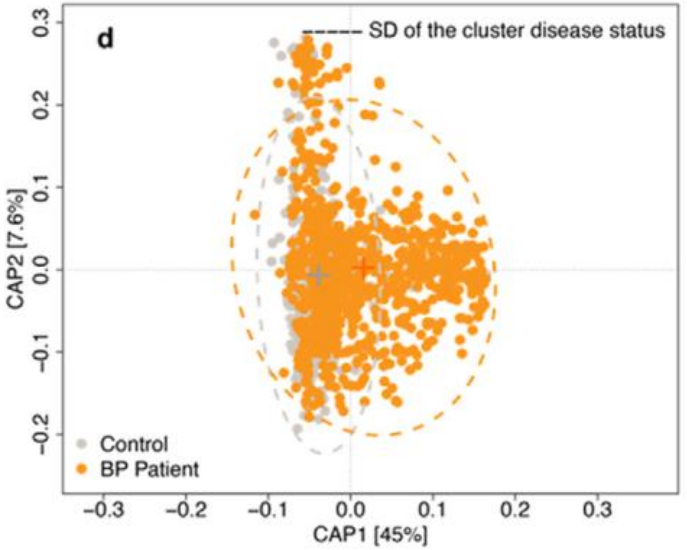


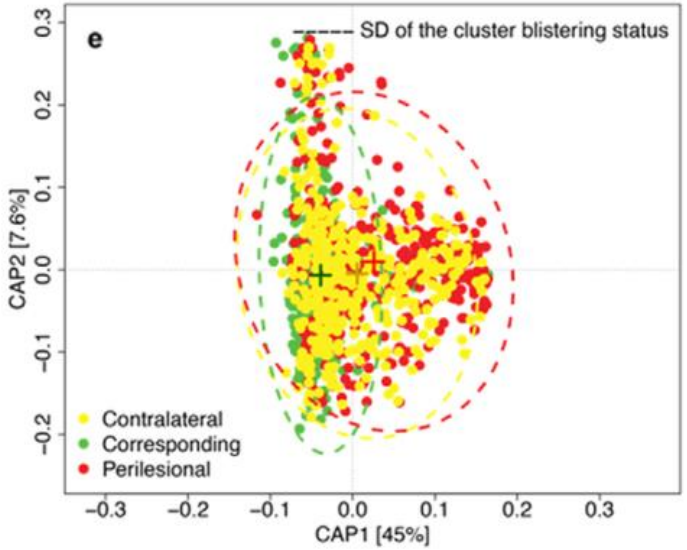


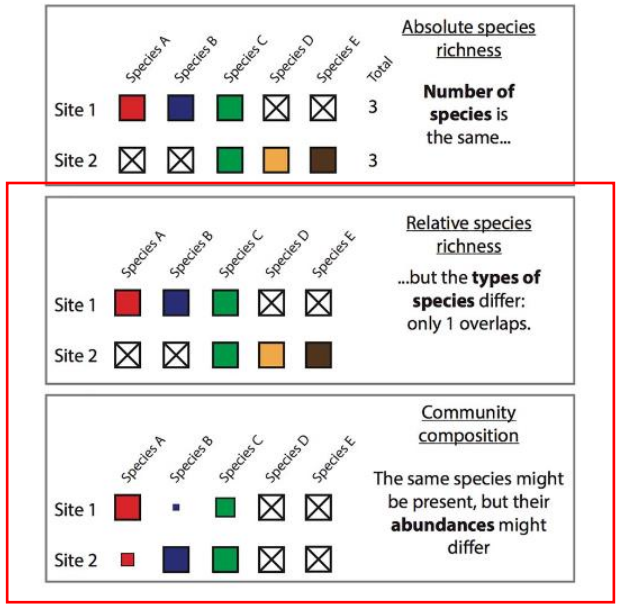

Species A
Species B
Species C
Species D
Species E
Total
Site 1
Site 2
3
3
Absolute species richness
Number of species is the same...
Relative species richness
...but the types of species differ: only 1 overlaps.
Community composition
The same species might be present, but their abundances might differ

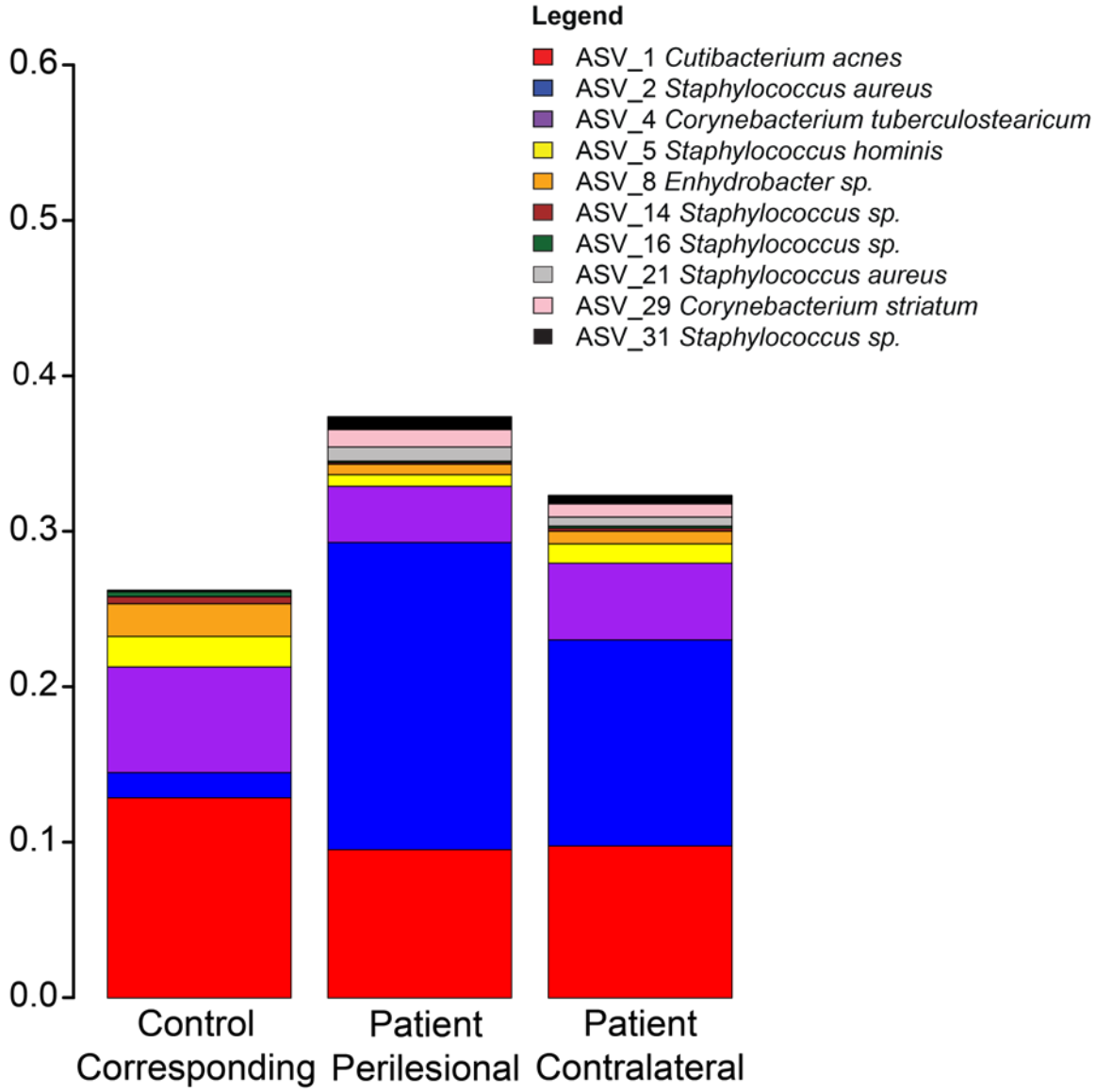

a
SD of the cluster disease status
Control
BP Patient
CAP2 [12.2%]
CAP1 [52%]


b
SD of the cluster body site
Antecubital fossa
Forehead
Upper back
CAP2 [12.2%]
CAP1 [52%]


d
SD of the cluster disease status
Control
BP Patient
CAP2 [7.6%]
CAP1 [45%]

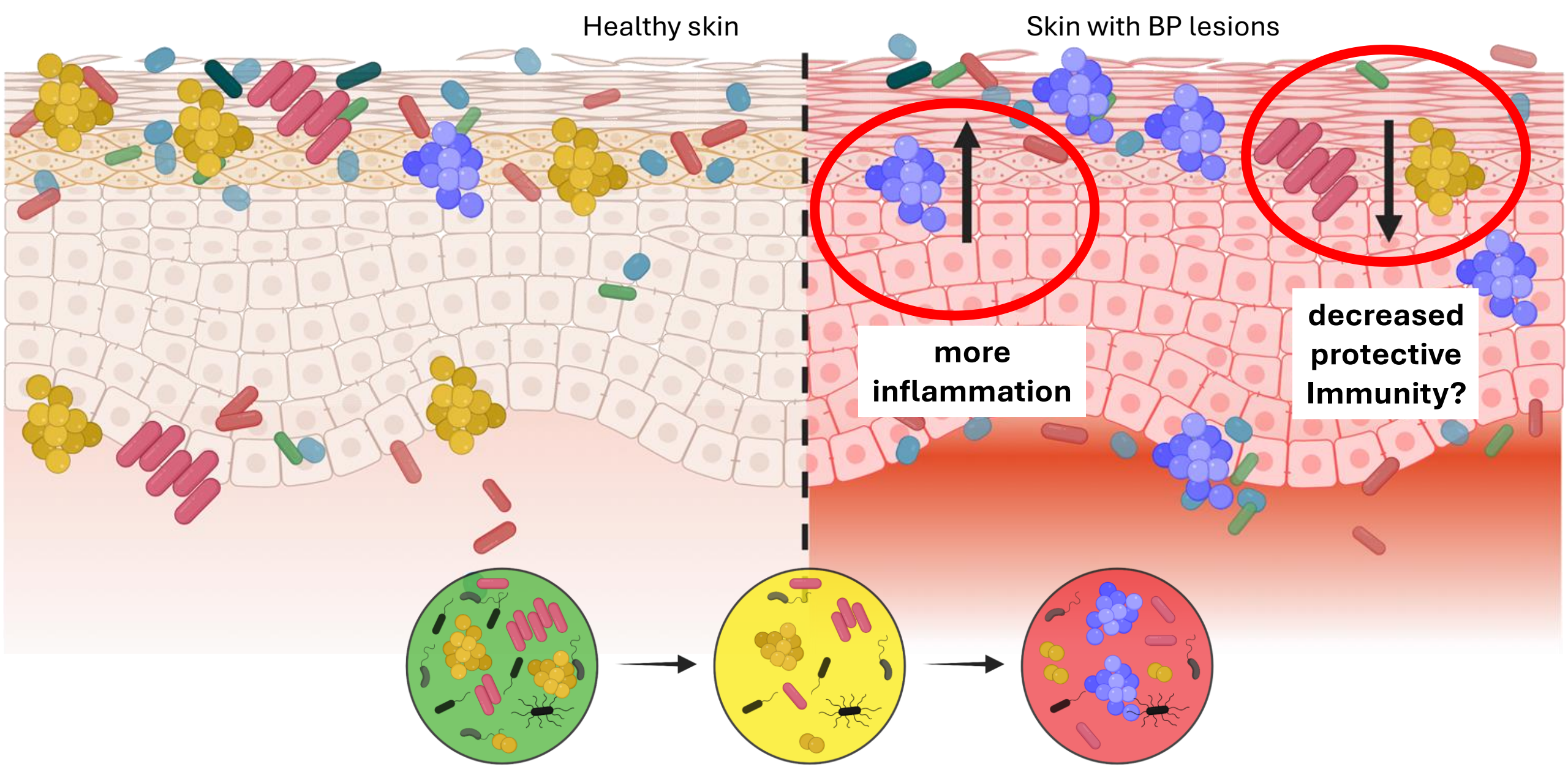

e
SD of the cluster blistering status
Contralateral
Corresponding
Perilesional
CAP2 [7.6%]
CAP1 [45%]





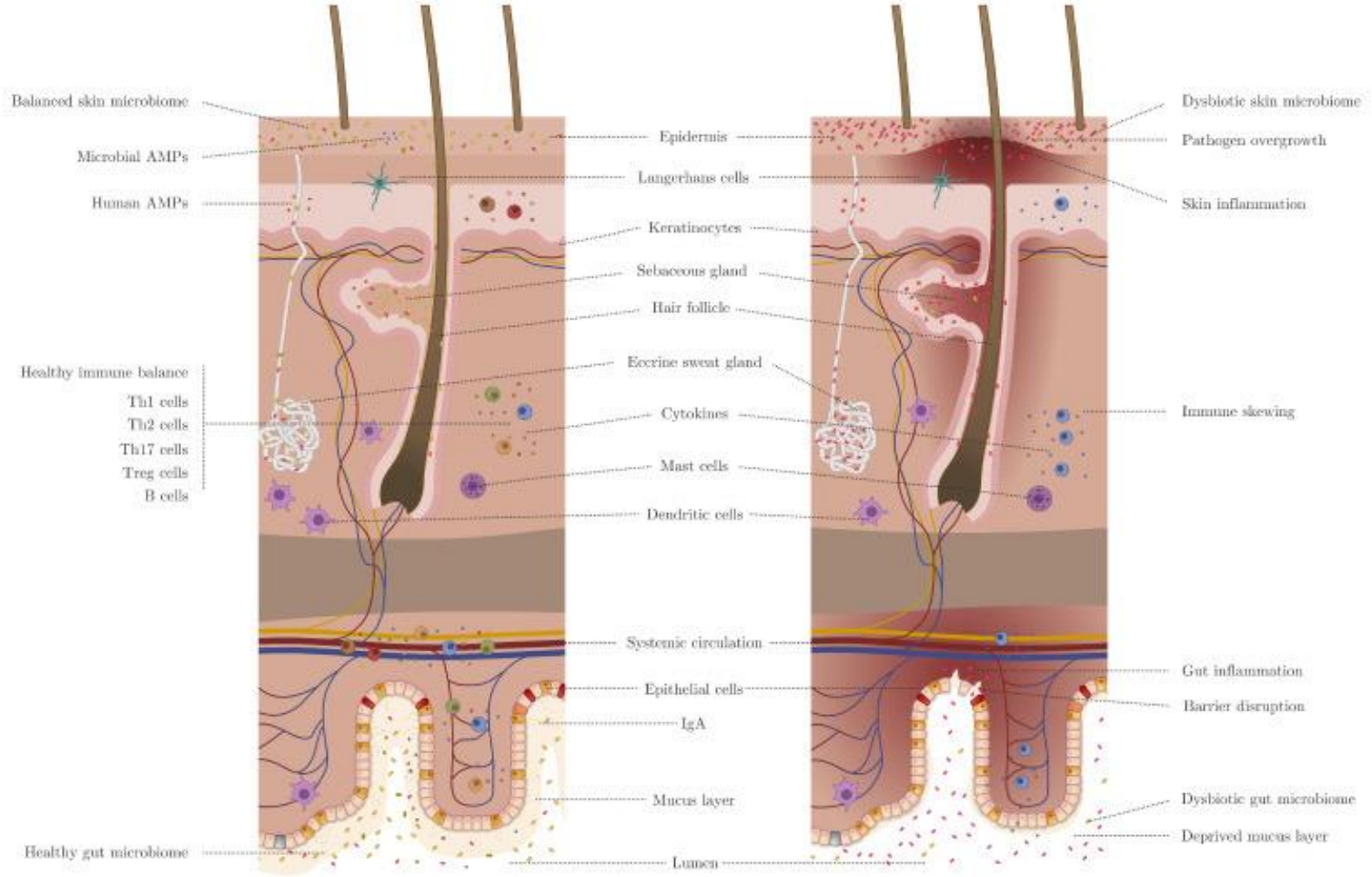
Disease severity & blistering associates with increased *S. aureus*

S. hominis is more abundant in controls and negatively correlates with disease status and with disease severity


Healthy skin
Skin with BP lesions
more inflammation
decreased protective Immunity?

What about Gut microbiome of these patients?

- Recent studies have highlighted a correlation between gut microbiome and skin **(gut-skin axis).**
- Some dermatoses are comorbidities of gastrointestinal disorders.
- Moreover, multiple inflammatory skin disorders, such as psoriasis and atopic dermatitis are accompanied by gut dysbiosis, including altered diversity and composition of the gut microbiota.
- Gut dysbiosis could increase host vulnerability and trigger an immunological response, resulting in skin imbalances.



Conclusions

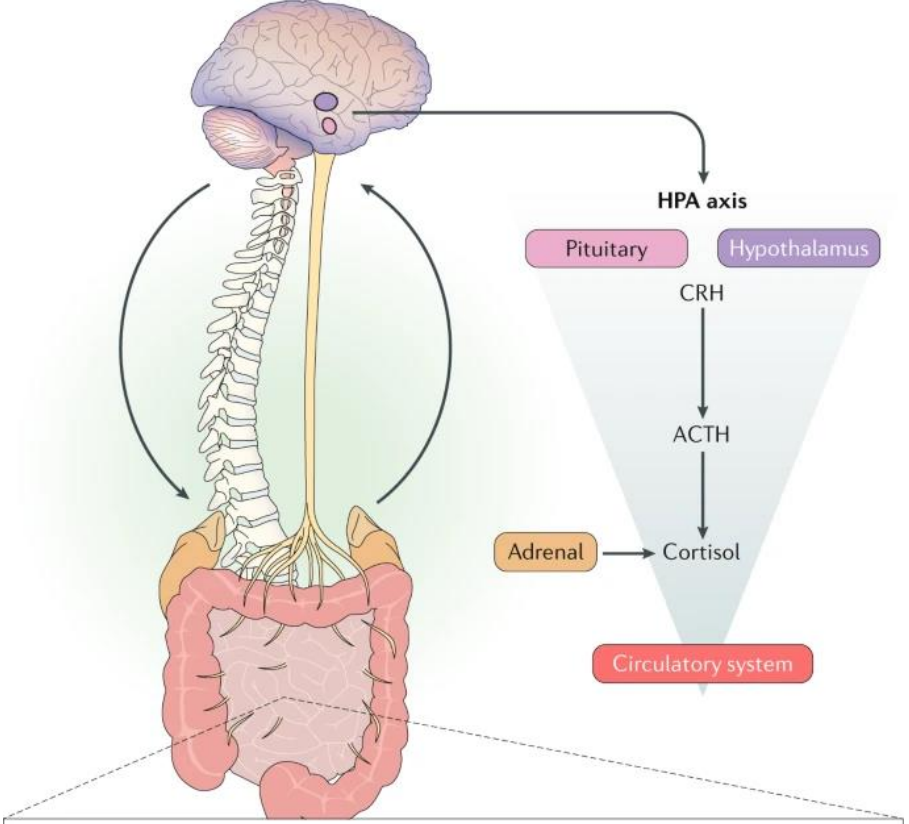
Limitations:

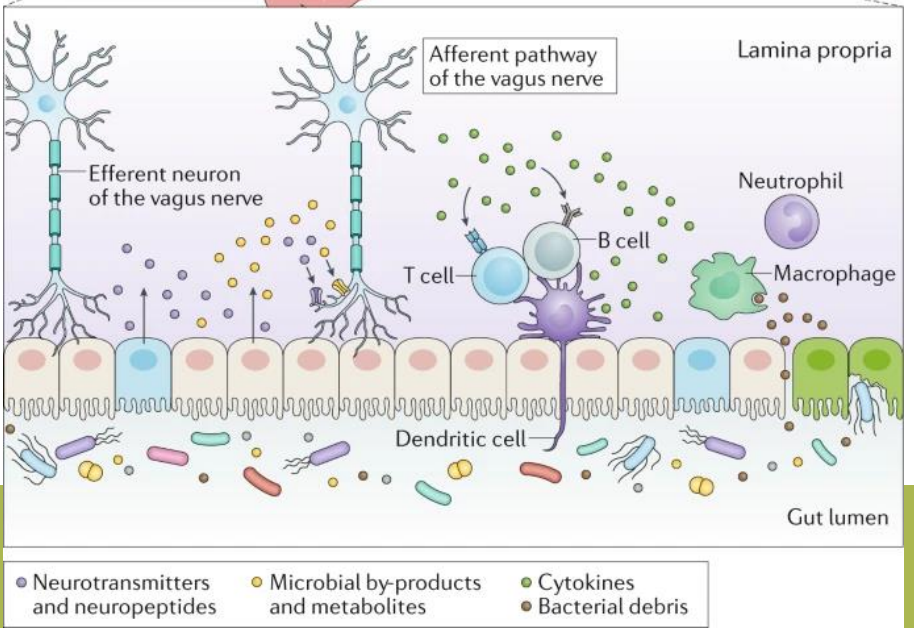
- comparatively small sample size and lack of longitudinal data.

Conclusions:

- dysbiotic features in the gut microbiome across inflammatory diseases
    - reduced alpha diversity, reduced *F. prausnitzii*, role for GABA-related pathways
- This study emphasizes the importance of the gut-skin axis.

- Future studies including longitudinal data and experimental preclinical models are thus justified to help establish causality and test microbiome-based intervention strategies.





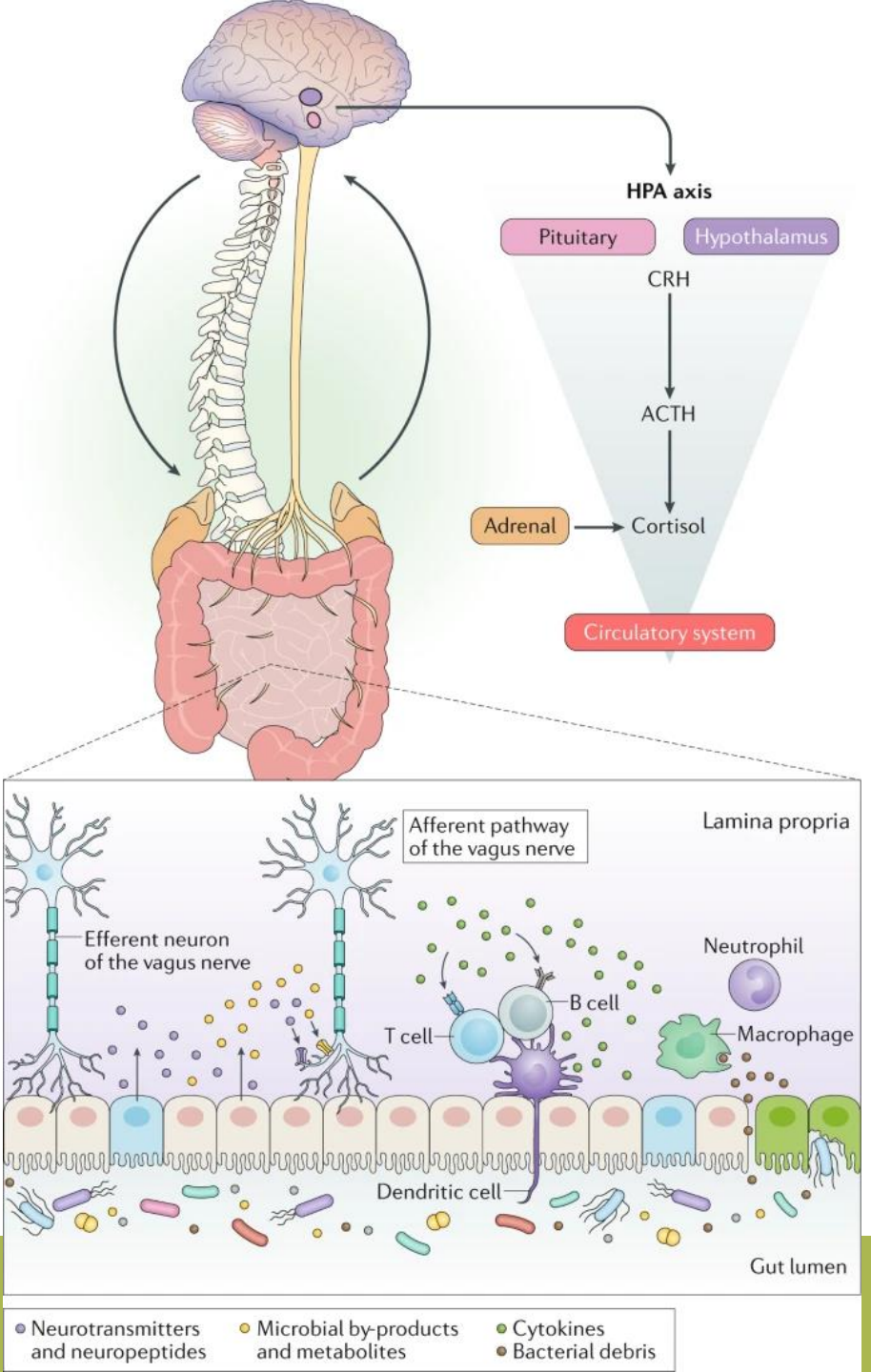
Introducing the GBA

The ‘gut–microbiota–brain axis’ refers to the **network of connections** involving multiple biological systems that allows bidirectional communication between gut bacteria and the brain.

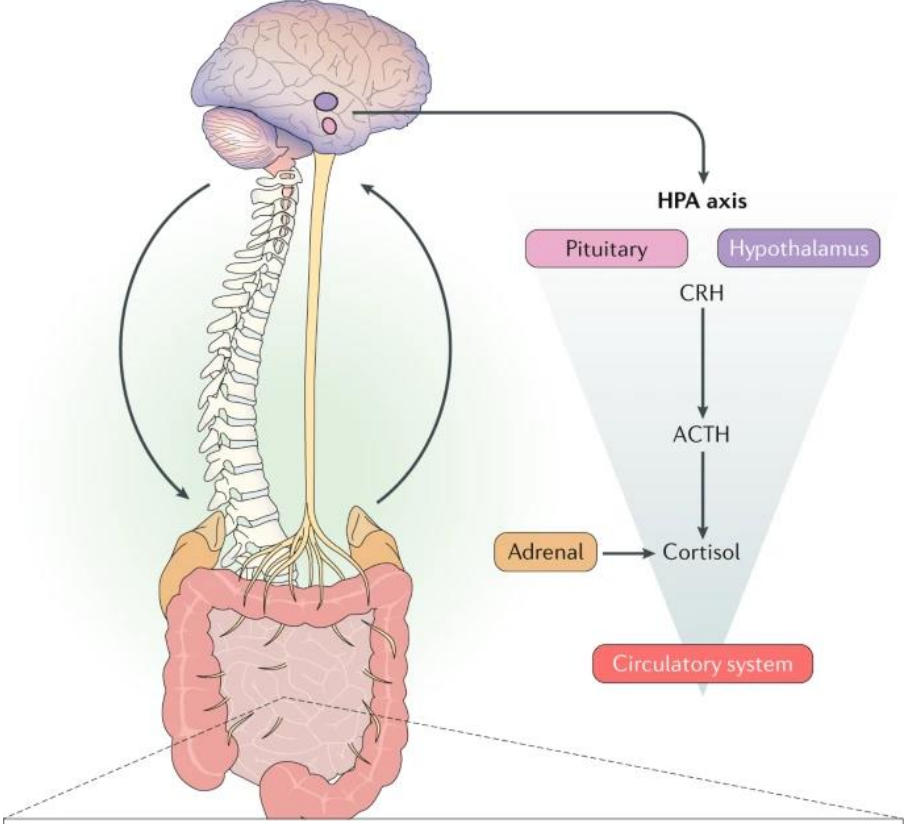
GBA involves both direct and indirect signalling via chemical transmitters, neuronal pathways and the immune system.

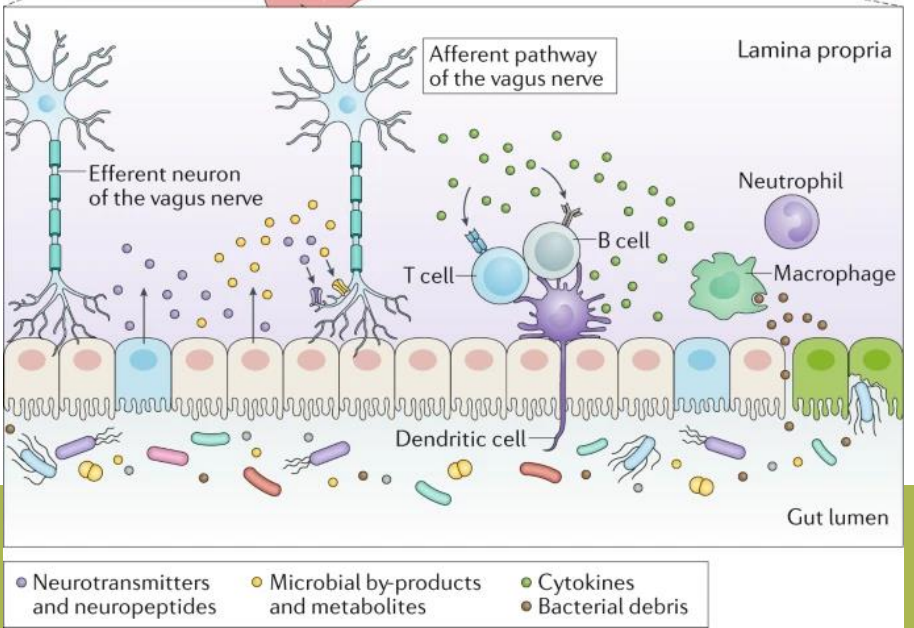
It involves the enteric nervous system (ENS) and the vagus nerve, the neuroendocrine system, the hypothalamic–pituitary–adrenal (HPA) axis, the immune system and metabolic pathways.

Gut microbiota:



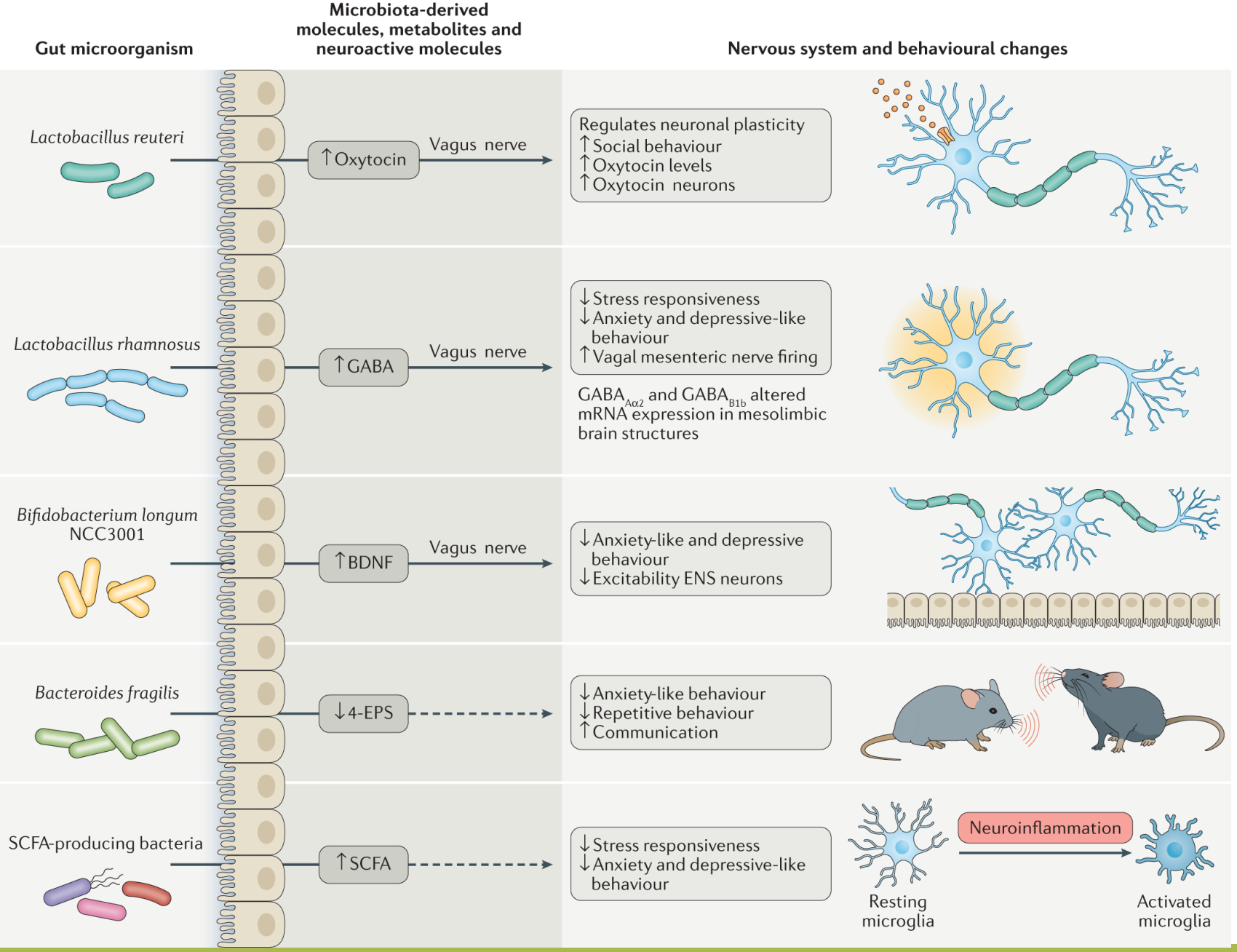
- can produce neuroactive compounds such as **neurotransmitters** (for example, γ-aminobutyric acid (GABA), noradrenaline, dopamine and serotonin (5-hydroxytryptamine (5-HT))), **amino acids** (for example, tyramine and tryptophan) and **microbial metabolites** (for example, short-chain fatty acids and 4-ethylphenylsulfate).
- These metabolites can travel through **portal circulation** to interact with the host immune system, influence metabolism and/or affect local neuronal cells of the ENS and afferent pathways of the vagus nerve that signal directly to the brain.
- The gut microbiota can also influence **gut barrier integrity** that controls the passage of signalling molecules from the gut lumen to the lamina propria, which contain immune cells and terminal ends of ENS neurons, or to portal circulation. Gut barrier integrity can become disrupted in some neuropsychiatric conditions, such as anxiety, autism spectrum disorder and depression.





The SNC:

- Stress can activate the HPA axis response that involves neurons of the hypothalamus that secrete hormones such as corticotropin receptor hormone (CRH) into the brain or the portal circulation, triggering the release of adrenocorticotrophic hormone (ACTH), which then initiates the synthesis and release of **cortisol**. Cortisol regulates neuroimmune signalling responses that, in turn, **affect intestinal barrier integrity**.
- Stress hormones, immune-mediators and CNS neurotransmitters can activate neuronal cells of the ENS and afferent pathways of the vagus nerve, which can **change the gut environment and alter the microbiota composition**.



- Oxytocin increases social behaviour.
- administration increases social behaviour in mouse models of autism spectrum disorder.

- attenuates depression and anxiety-like behaviour in mice but failed to improve stress symptoms in healthy humans

- ameliorate mood alterations
- reduces anxiety and depression-like behaviours in mice

- is known to improve anxiety-like behaviour, repetitive behaviour and communication in mice

- regulate genes that are involved in microglia maturation

Anorexia nervosa (AN)

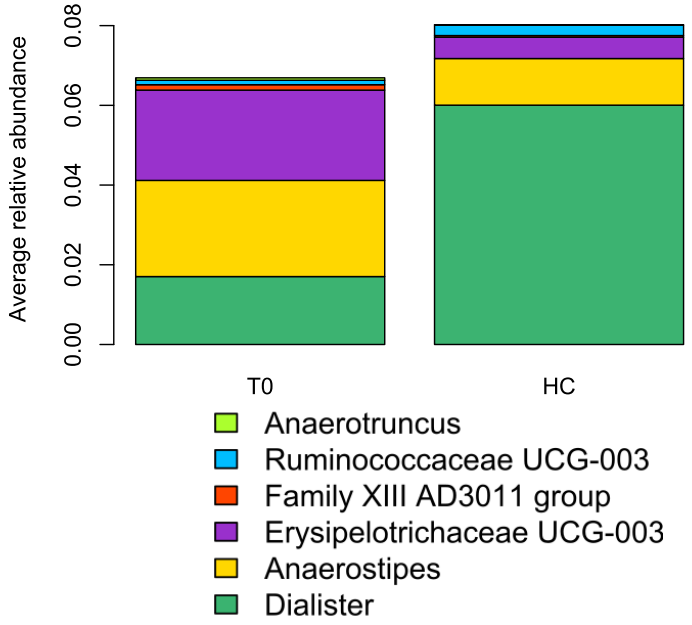
- is the third-most common chronic disease in adolescence and the deadliest of all psychiatric diseases with a standardized mortality rate 5-10 times higher than in healthy controls
- is characterized by insufficient energy intake and thus low body weight, body image distortion and fear of gaining weight.
- Its underlying pathophysiology is poorly understood.
- Treatment includes weight restoration and psychotherapy but often remains inadequate and there is a high rate of relapse.
- Peak onset age 13-18 y
- Lifetime prevalence 0.5% - 2.0%

Anorexia nervosa (AN)

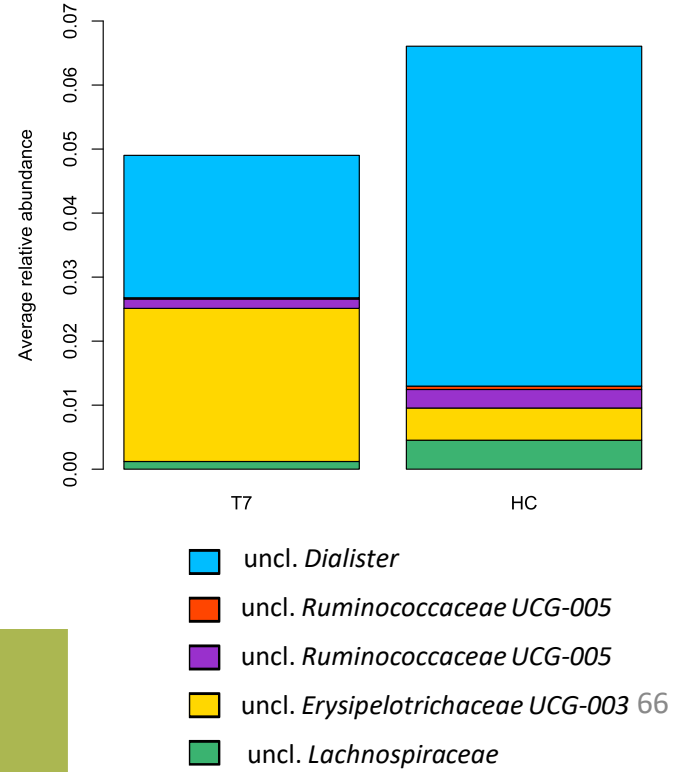
- Gut microbiome is increasingly recognized as an influencing factor for energy extraction from food , weight regulation, as well as the influence on the brain and behavior via the gut-brain axis.
- Patient studies during acute starvation all confirm intestinal dysbiosis, albeit with heterogeneous results
- Identifying influencing factors and taxa relevant for prognosis, might be crucial to better understand the underlying host-microbe interactions and their role in the pathophysiology of AN.
- Identifying the taxa involved in the development and maintenance of the disease might allow for screening of patients at disease onset and prediction of their clinical course.
- Identifying bacteria associated with healthy gut could advise on the implementation of a probiotic treatment.

… and specifically

- Also in Schulz et al., the genus *Anaerostipes* is significantly more abundant in patients at admission when compared to HC.
- *Anaerostipes* is suggested to regulate human behavior. This genus is increased in psychiatric disorders such as depression and bulimia nervosa (Leyrolle et al., 2021).
- Members of the genus *Anaerotruncus* (mucin-degrader) has been reported to be higher in nutrient-deprived ecosystem (Crost et al., 2013).



- Ruminococcaceae family has been reported as being more abundant in HC vs AN patients (both in Specht et al., 2022 & Borgo et al., 2017).
- Lower abundance of *Dialister* spp. has been reported to correlate to anxiety disorder (Garcia-Gil et al., 2022).
- Members of the *Erysipelotrichaceae* positively correlate with levels of TNF-alpha (that we know are significanlty more abundant in T0P; Specht et al., 2022).

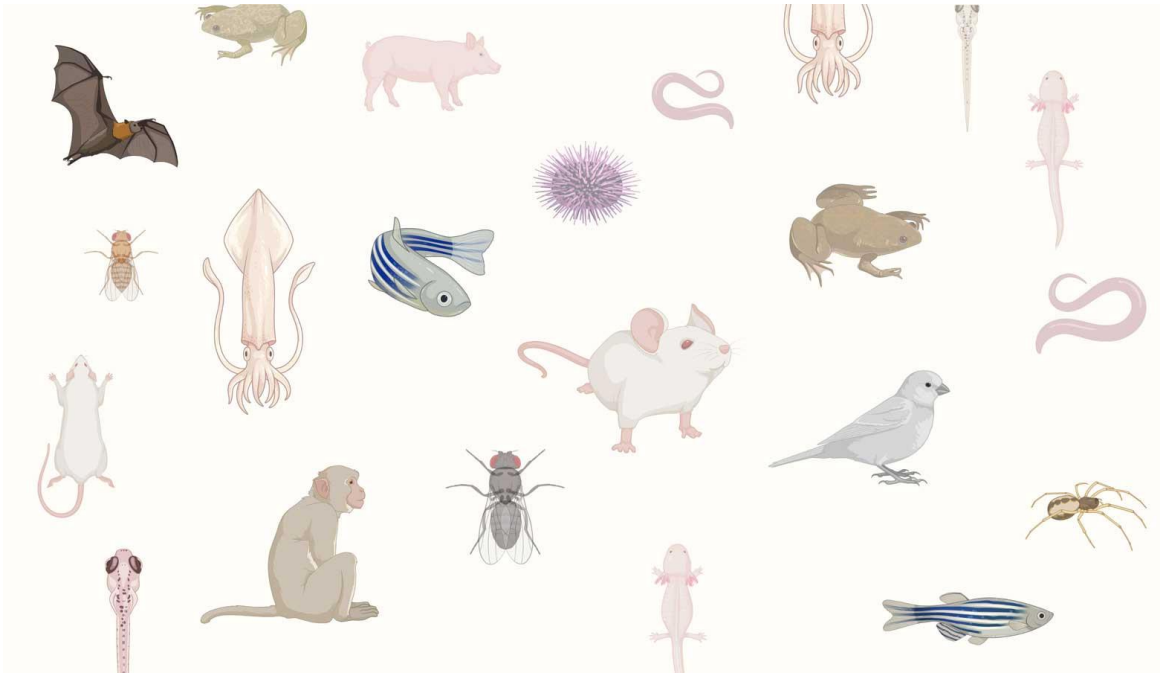


Conclusions

- we showed diminished, yet ongoing alterations in the gut microbiome after 1 year follow-up even in weight recovered patients
- We identified taxa that predicted the clinical course could help to distinguish patients at admission and increase therapy intensity where most needed
- *Sutterella* and Lachnospiraceae uncult could be promising probiotic supplements to be tested as future microbiome-targeted additions to existing AN treatment

Animal models

- A non-human species used in biomedical research because it can mimic aspects of a biological process or disease found in human.
- Commonly used animal models comprise a variety of species.
- The choice of animal species depends on the what you want to study
- To date, maximum microbiome research has been focused on the **mouse as a model** organism for studying the mechanisms of different processes occurring in the microbial communities.
- Other models: zebrafish, pigs, and *Drosophila.*



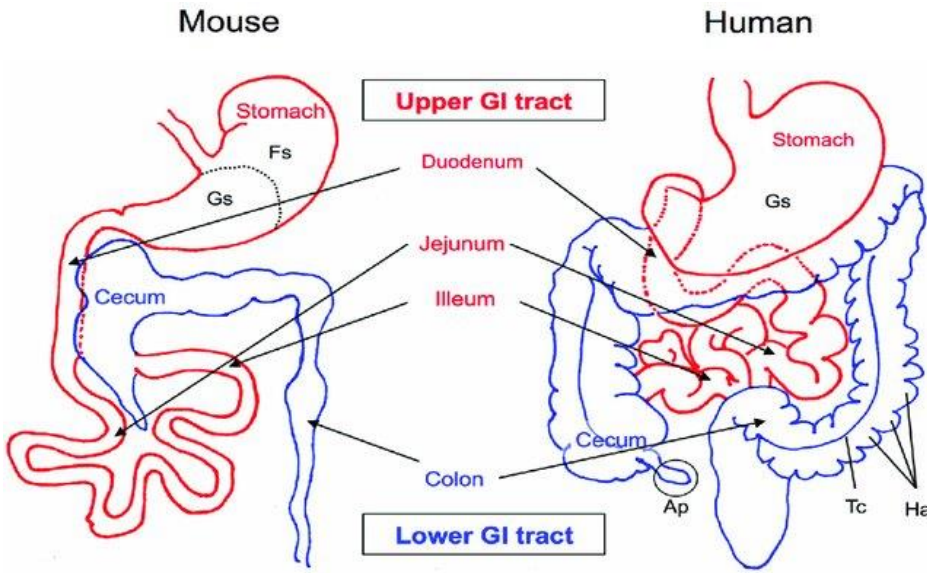
Mouse

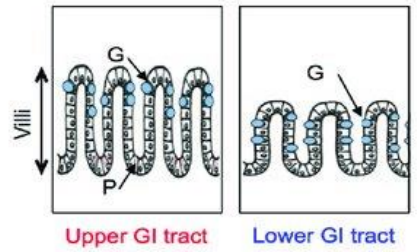
- Different genotypes/phenotypes available (more than any other models).
- Physiology and anatomy of mice are similar to humans.
- GI especially (anatomically similar organs)
  - Cecum is larger in mouse
  - Colon in mouse has a muscolaris mucosae

- Mice share around 99% genes with humans.
- key similarities with human gut microbiome at phylum through family level.

Mice could be SPF or GF.

ADVANTAGE: very controlled genetic background / environmental conditions / small size.





GF mice

- Mice that have no bacterial load.

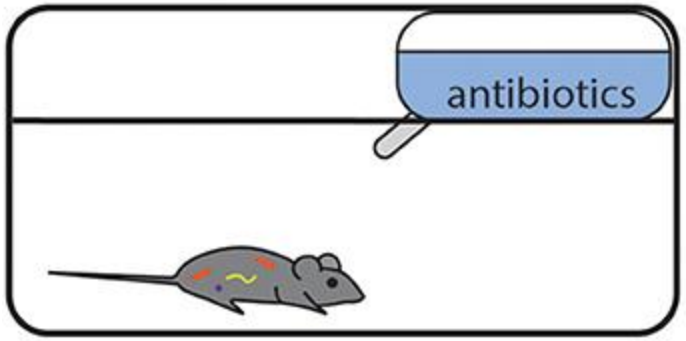
- An embryo is created through in vitro fertilization and then transplanted into a germ-free mother.
- If this method is not available, a mouse can be born through cesarean birth, but this comes with a higher risk of contamination.

- Usually these animal are healthy, with cecum a bit bigger than the usual, as bacteria of the gut cannot ferment the food
- They are fed with sterile food, they drink sterile water.
- The bedding is sterile, as well as all the equipment used.

- GF mice enter the experiment when they are transplanted with single strains or a complex community.

GF vs Microbiome Depleted Animals

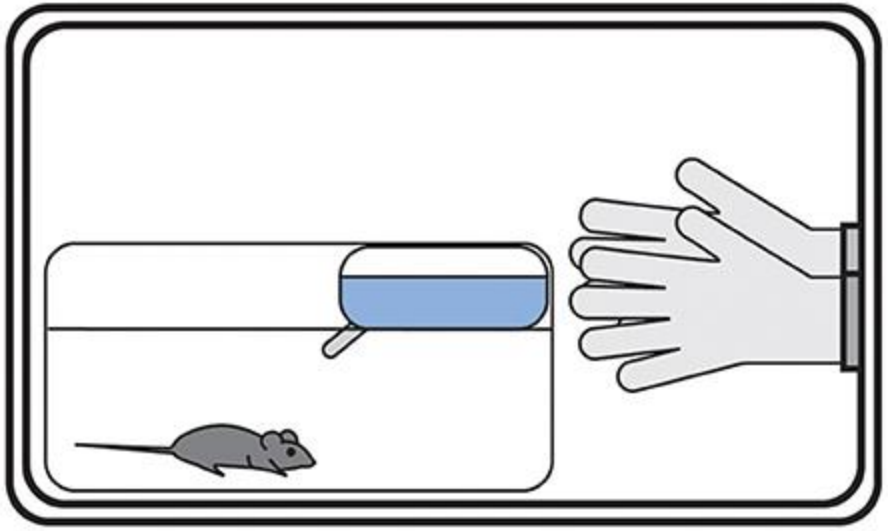
Antibiotics Treatment



Pros:
- Inexpensive
- No specialized equipment needed
- Applicable to any genotype

Cons:
- Some bacteria still present
- Other microorganisms still present
- May affect eukaryotic cells
- May select for resistant bacteria or promote fungal outgrowth

Germ-Free Conditions

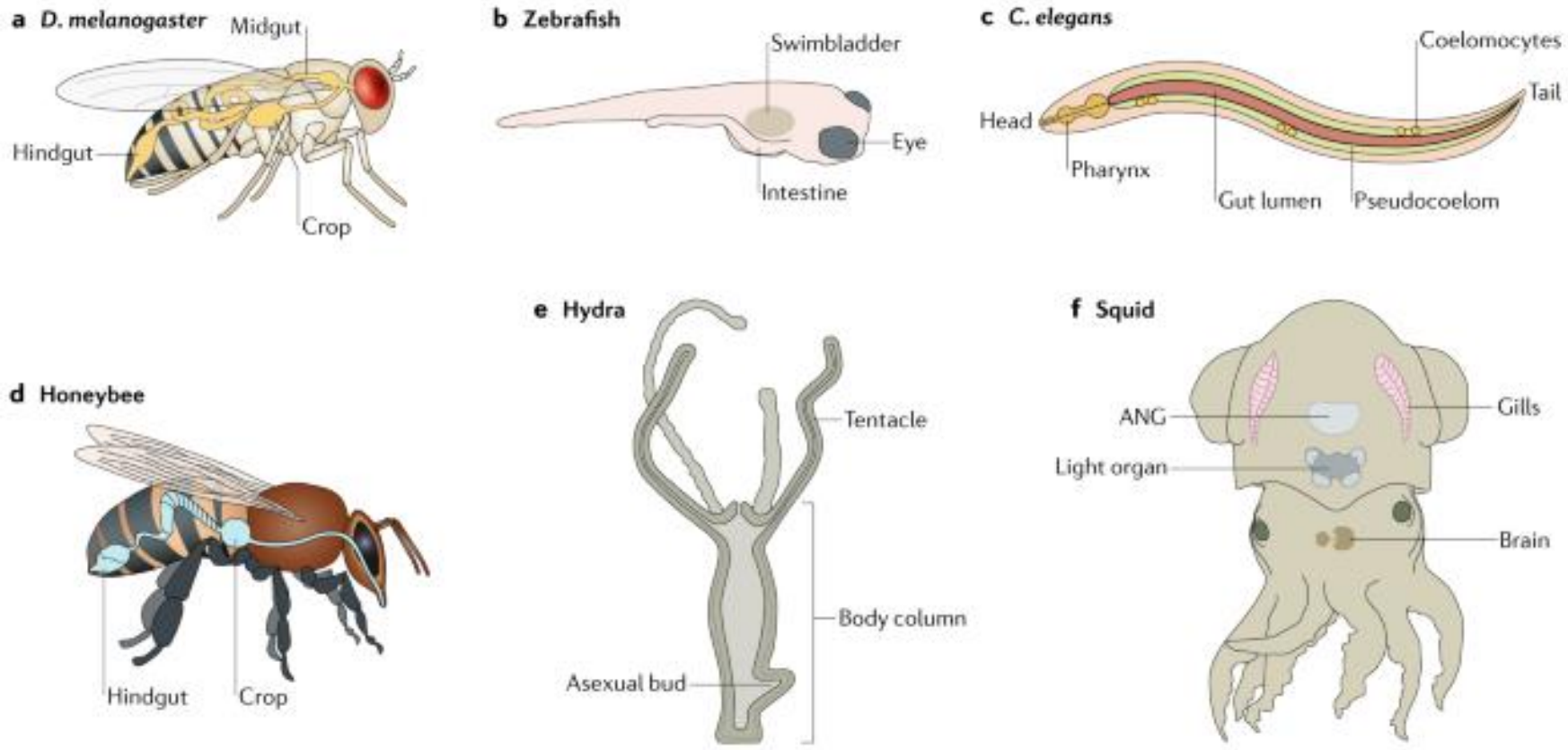


Pros:
- Mice are free of all microorganisms, in all tissues
- Allows for exclusive colonization with defined microbes

Cons:
- Expensive
- Requires specialized equipment and training
- New genotypes must be re-derived
- Not all experiments feasible
- Developmental defects

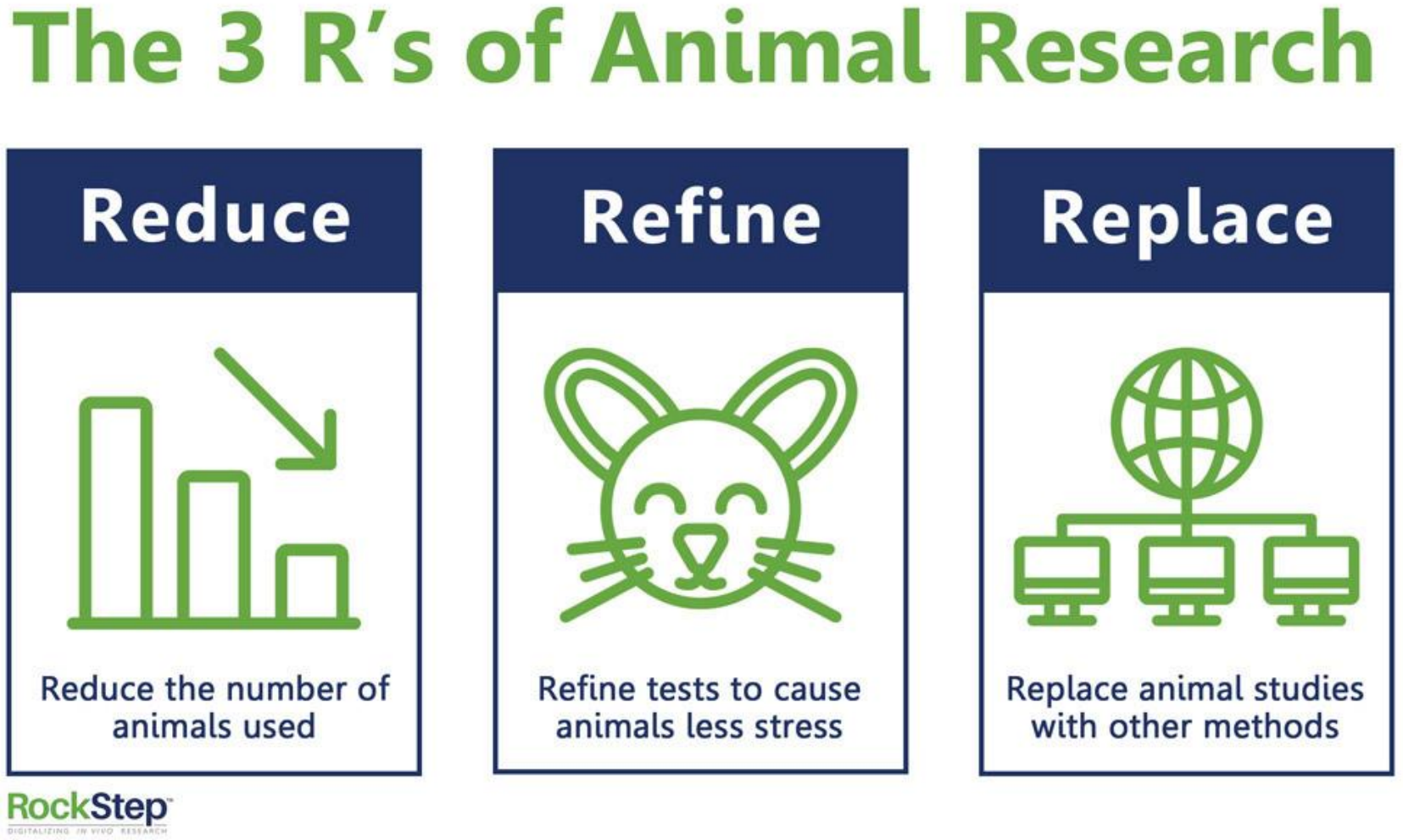
Simple animal models for microbiome research

- lower vertebrate and invertebrate species with low diversity microbiomes
- more cost-effective and time-efficient than mammal models, especially for complex experimental designs and sophisticated genetic screens.



https://doi.org/10.1038/s41579-019-0242-1

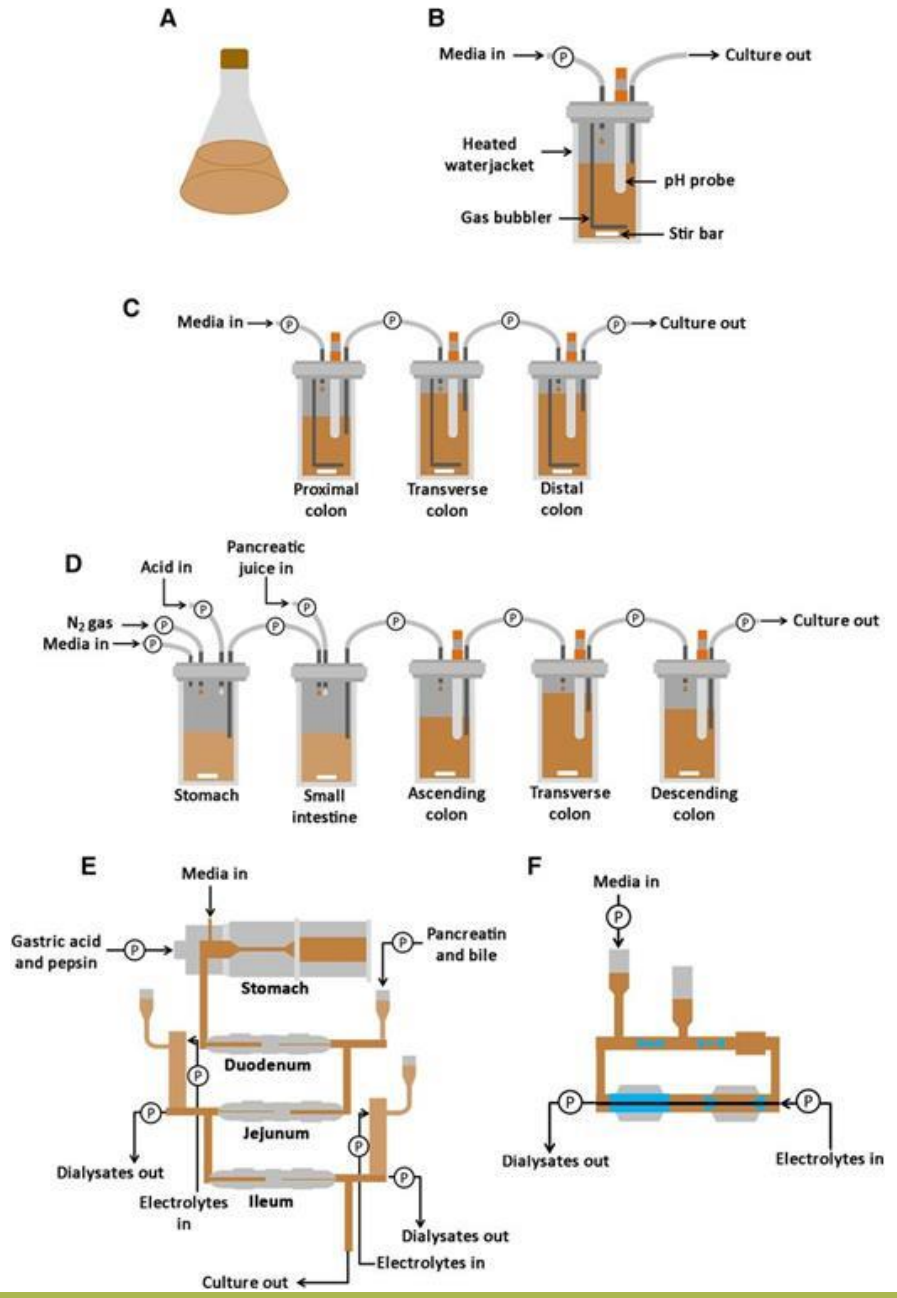
Directive 2010/63/EU and the 3Rs



Alternative models:

Gut fermentation models

- can recapitulate spatial, temporal and environmental features
- Usually operated under anaerobic conditions
- High stability, very controlled
- Easy to induce a perturbation
- Can sample as many times you want
- Different options:



Alternative models:

- Less expensive than an animal
- Easy to handle and introduce perturbations
- More “acceptable” from the ethical point of view

- But:
- More distant to the human body

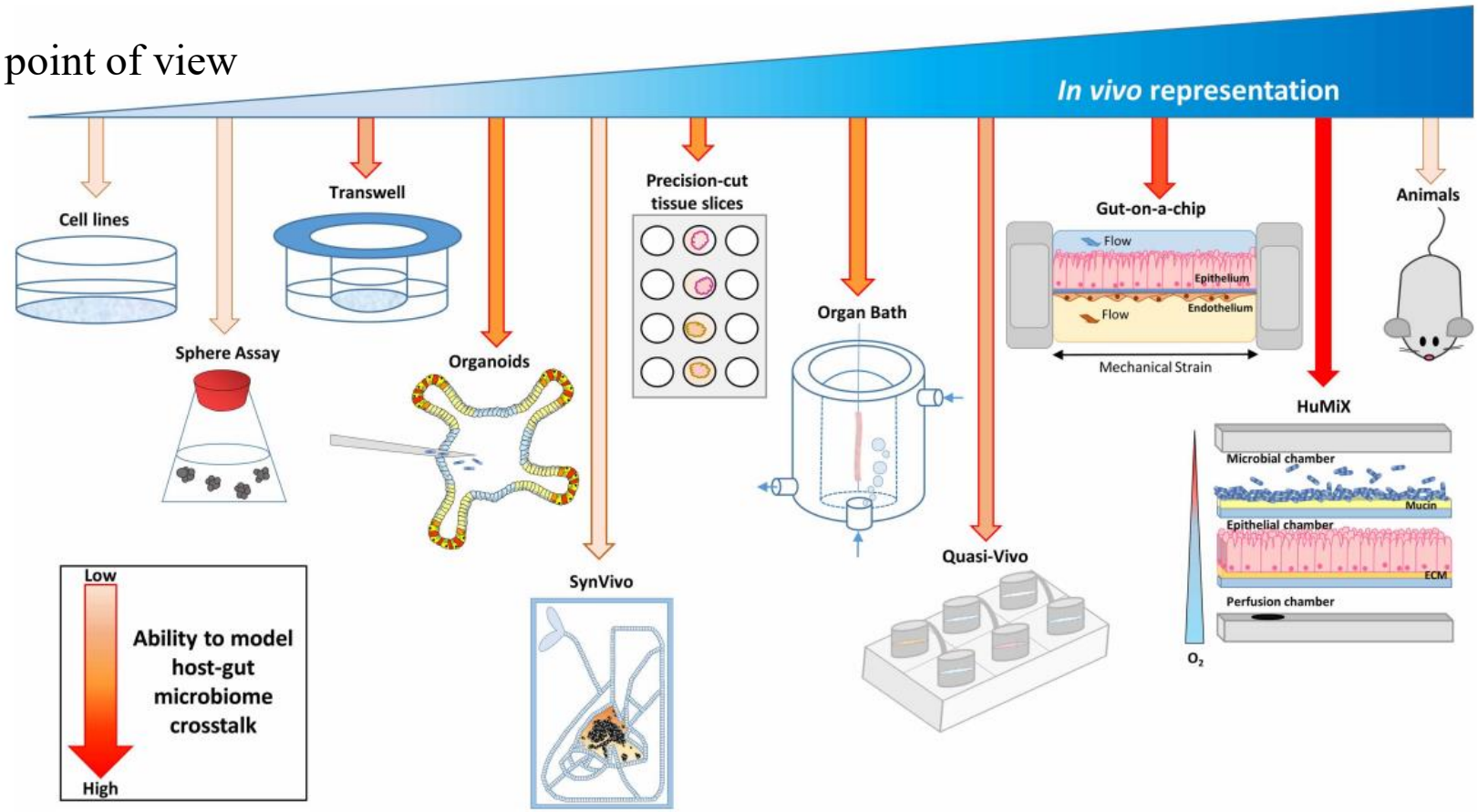
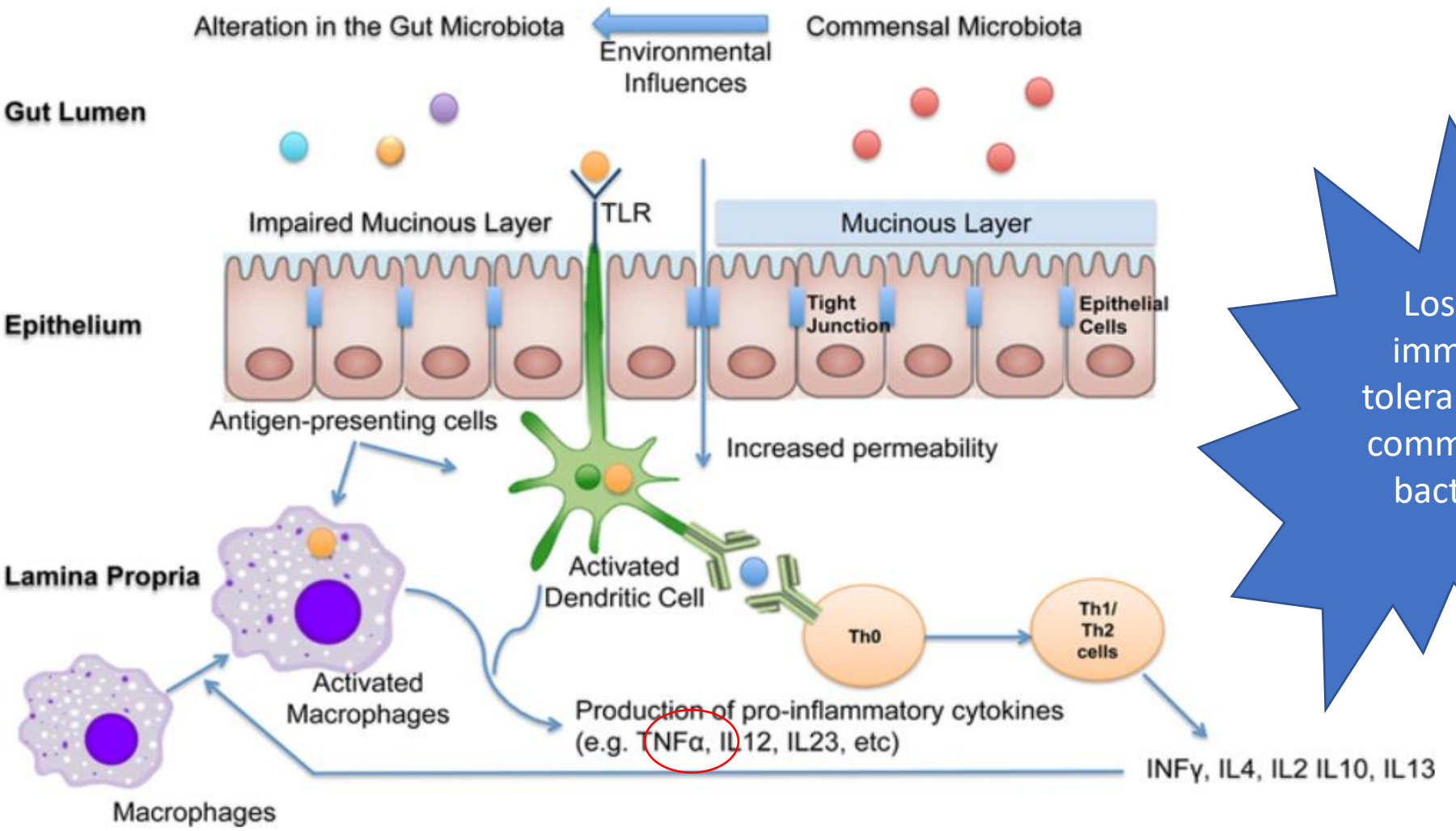


Figure 1. Current cell modelling systems and their potential to model host-microbiome cross-talk.
Schematic representation of current cell culturing techniques available and their ability to model host–microbiome interactions.

Inflammatory Bowel Disease (IBD) is a chronic, relapsing inflammatory condition of the GI.



Loss of immune tolerance to commensal bacteria

Khalili H et al. Nat Rev Gastroenterol Hepatol. 2018;15(9):525-535. doi:10.1038/s41575-018-0022-9

Inflammatory Bowel Disease (IBD) is a chronic, relapsing inflammatory condition of the GI.

✓ Multiple effective therapeutic options

✓ A proportion of patients fail to respond or lose response to therapy

Therapeutic options:

✓ Aminosalicylates

✓ Steroids

✓ Immunomodulators

✓ Anti-TNFα

20-40% of patients are primarily unresponsive
23-46% lose their response

Predictors of efficacy

Patient-related factors
• Age (earlier age at the start of IFX was associated with better outcomes in patients with CD)
• Weight (weight below 82 kilograms was associated with increased rates of clinical remission in UC patients treated with ADA)
Disease-related factors
• Disease duration (shorter disease duration was associated with increased efficacy in CD patients)
• Disease severity (disease severity was associated with worse therapeutic outcomes in UC patients)
• Disease phenotype (inflammatory phenotype is a predictive factor of response to anti-TNF-α than a complicating phenotype)
• Laboratory (biological) factors
√ High baseline CRP levels are predictive of response to anti-TNF-α in CD patients
√ High baseline Hb levels are associated with better response in UC patients
√ Low serum albumin levels are negatively correlated with response to anti-TNF-α in UC patients
Treatment-related factors
• Early clinical response ((i.d. within 3 months from starting therapy) is a predictive factor of long-term response in patients with UC)
• Mucosal healing (predictive factor for better therapeutic outcomes both in CD and UC patients)
• Trough levels of anti-TNF-α (anti-TNF-α serum concentration is directly correlated with better therapeutic outcomes in IBD patients)
• ATI (sustained high levels are associated to LOR)

IFX: infliximab; CD: Crohn's disease; UC: ulcerative colitis; ADA: adalimumab; ATI: Antibodies to IFX; IBD: inflammatory bowel diseases; LOR: Loss of Response.

Lopetuso LR et al., *Int J Mol Sci*. 2017;18(9):1973.

Aims:

✓ Create a mouse IBD model

✓ Test different anti-TNFα treatments

GF C57BL/6

n = 15
2 donors with UC

Patient 1: responder to anti-TNF-α therapy

P1 before treatment

P1 after treatment

Patient 2: non-responder to anti-TNF-α therapy

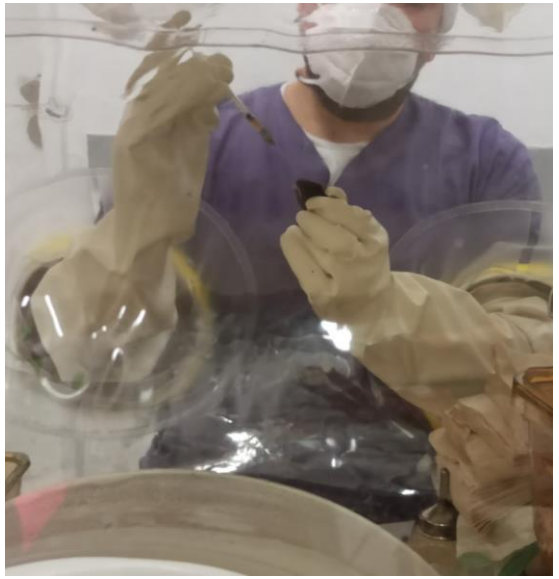
P2 before treatment

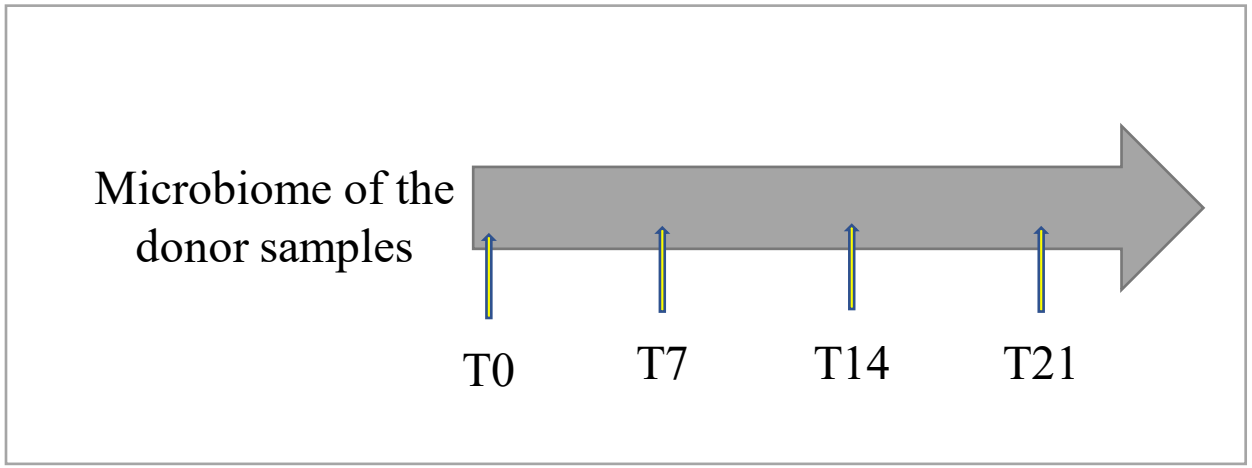
Trial experiment

GF C57BL/6

n = ~~15~~ 14

2 donors with UC





- ✓Mouse weight checked every 3-4 days
- ✓DNA extracted with PowerFecal DNA
- ✓Lipocalin measure with Mouse Lipocalin-2/NGAL DuoSet ELISA kit
- ✓CXCL1/KC in serum Mouse CXCL1/KC DuoSet ELISA
- ✓Microbiome investigated with 16S amplicon sequencing V3V4
- ✓Histopathology

Summary

- Gavage was successful in transferring the phenotype in the gnotobiotic mice
- GF WT B6 mice are a good model for UC, but there are some differences depending on donors
- Predicted metabolic functions were significantly different when comparing donors (independently from human vs mice), but not species (independently from donors)

Take home messages…

- you tell me!

(At least 10 things you learnt in these 3 hours together).

BAINES LAB

SECTION EVOLUTIONARY MEDICINE

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“Knowledge of sequences could contribute much to our understanding of living matter.”