Il Microbiota intestinale

nella salute e nella malattia

Dott.ssa Federica Del Chierico

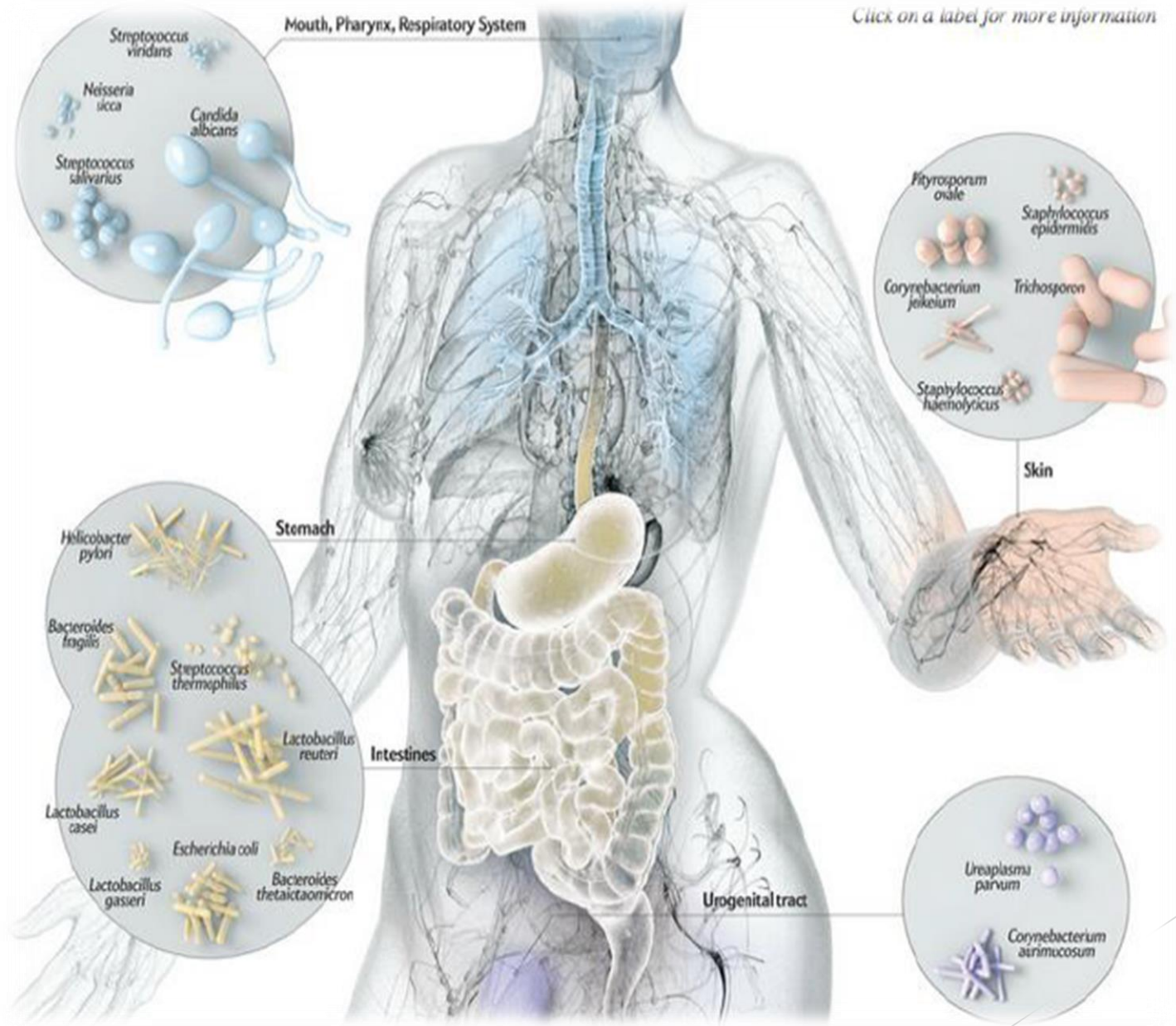
Unità di microbioma umano

Ospedale Pediatrico Bambino Gesù

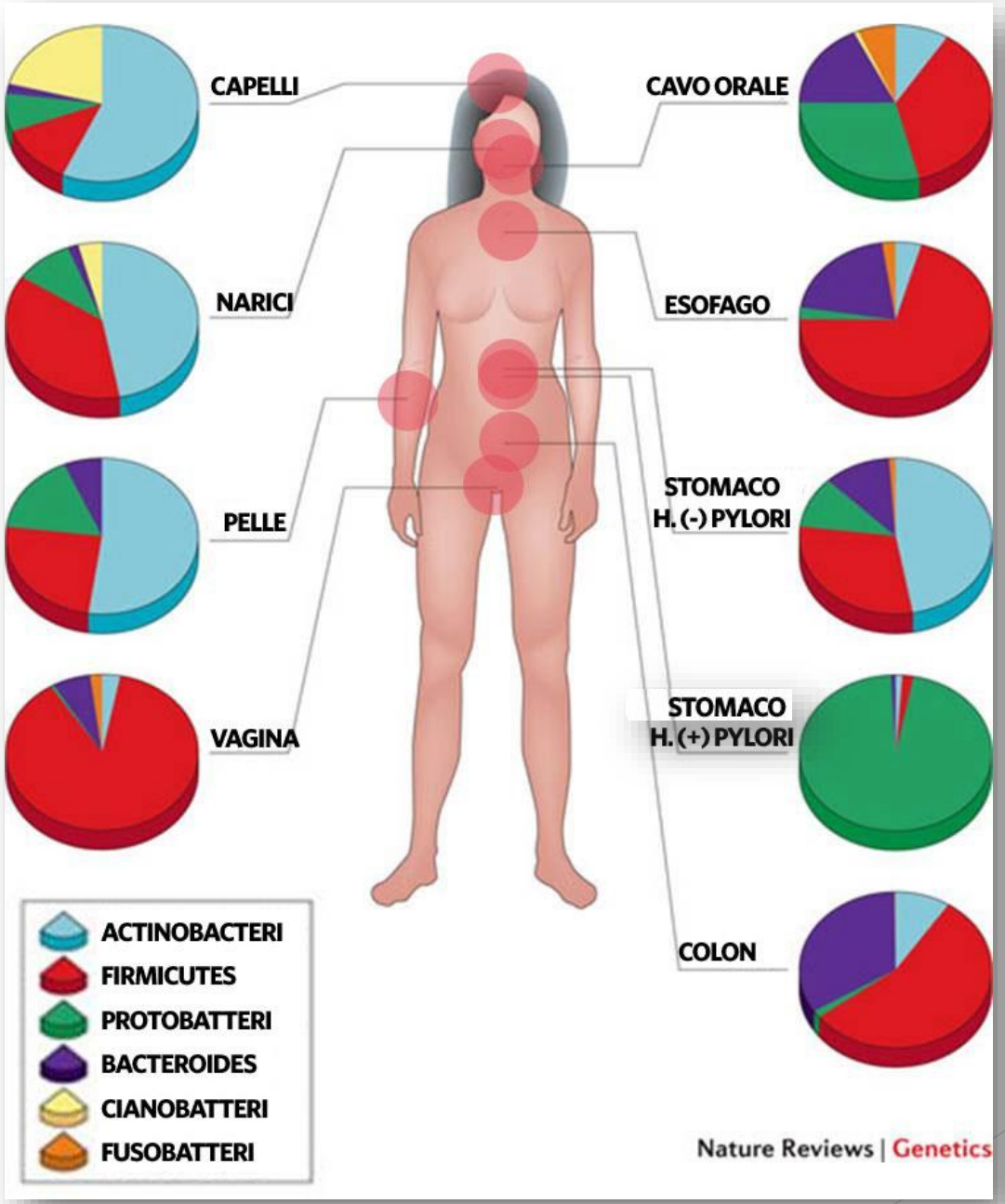
Tutte le malattie hanno origine nell’intestino…

Ippocrate 460 a.C. – 377 a.C.

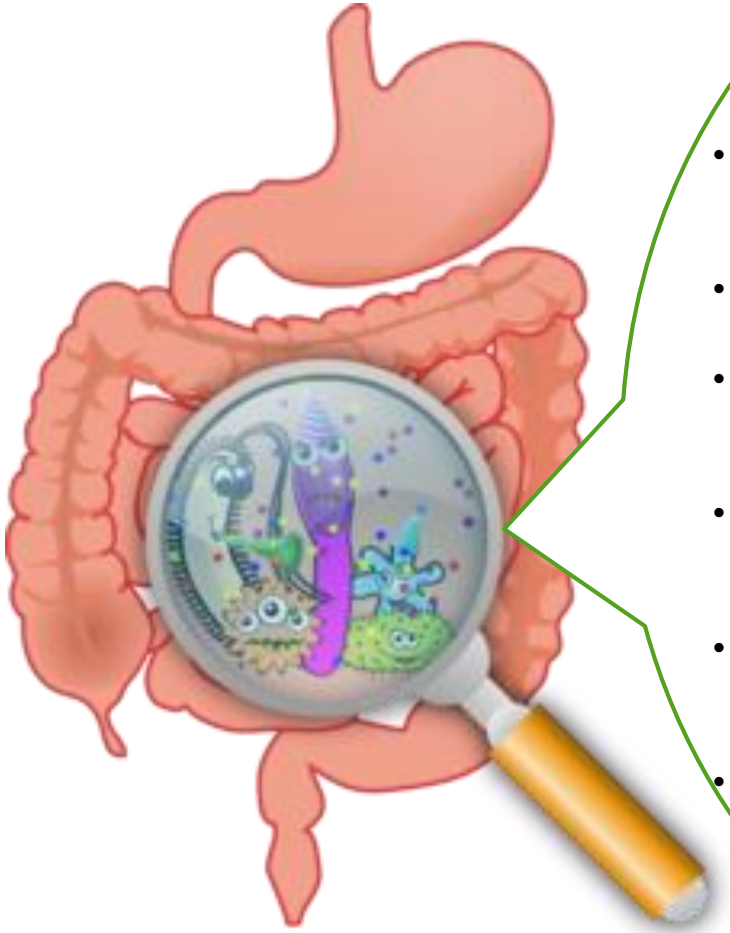
Il microbiota



Il microbiota



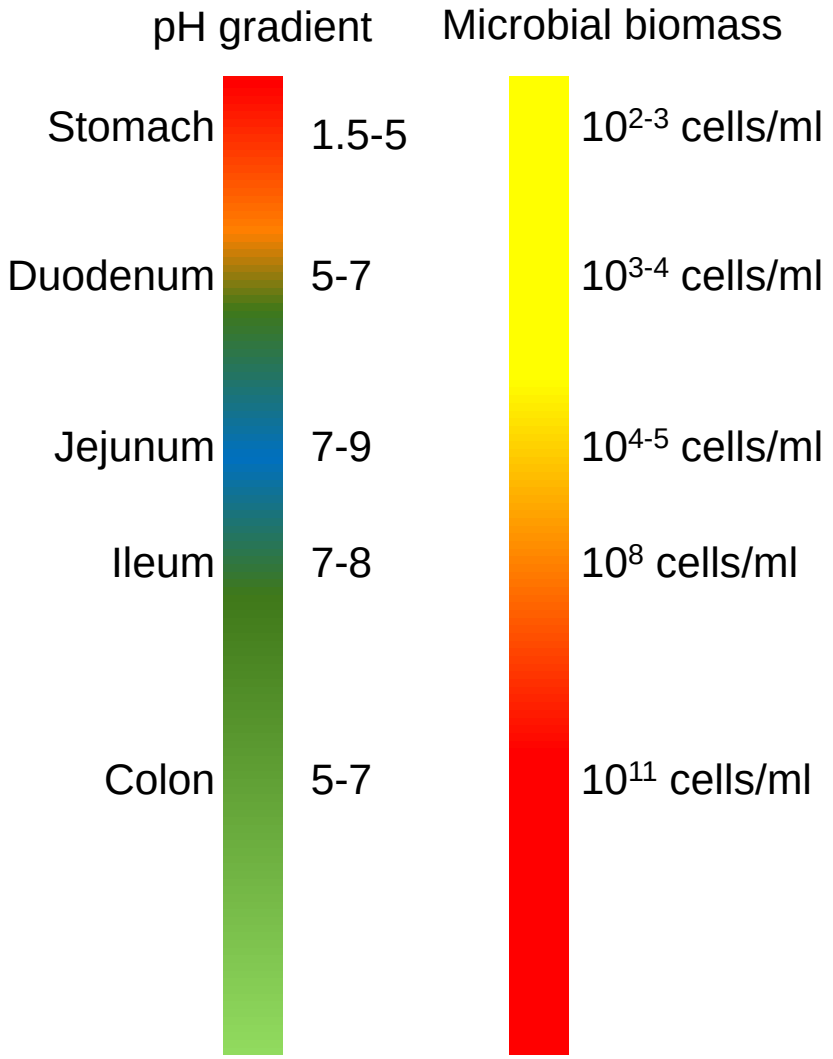
Il microbiota intestinale

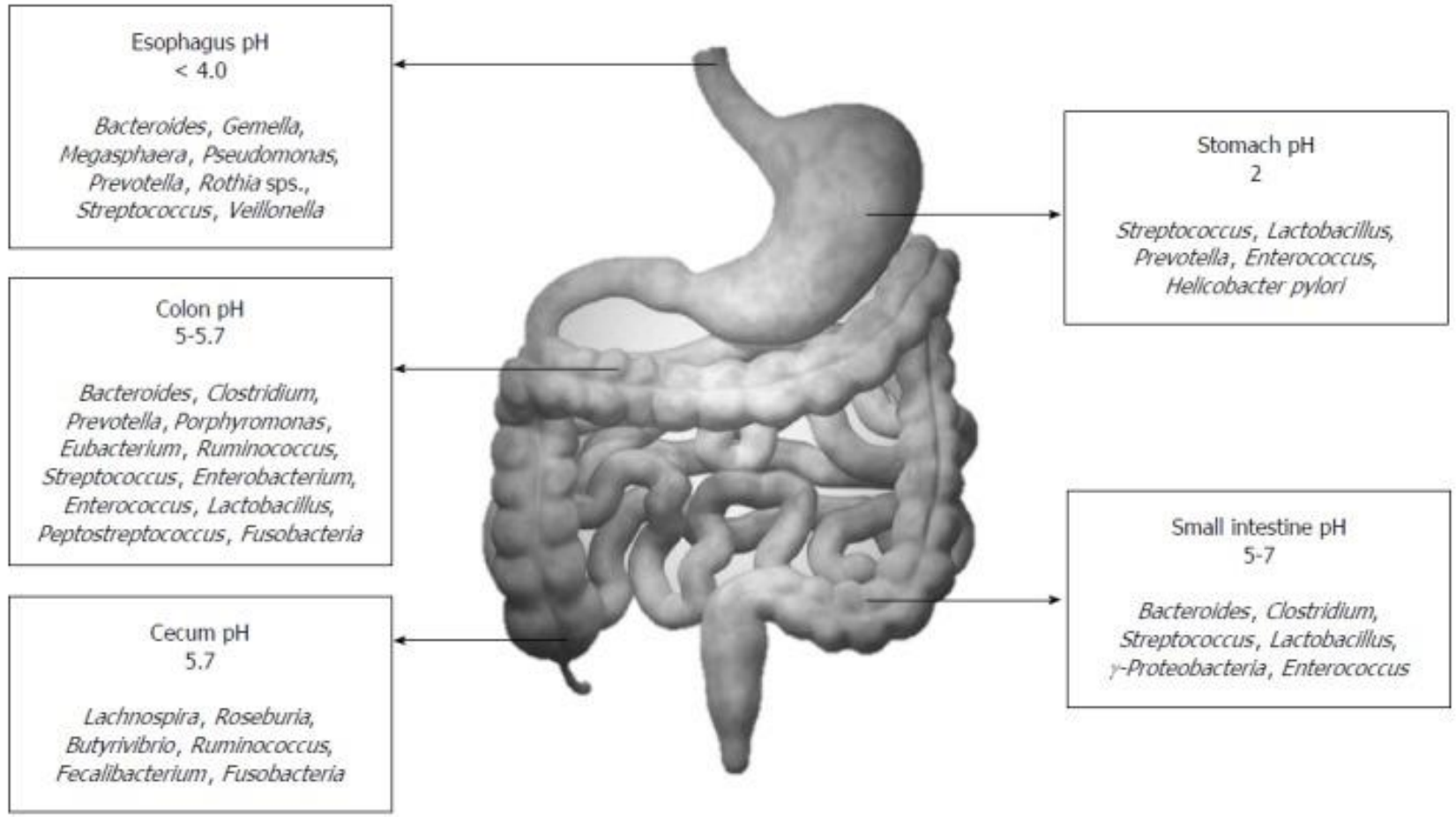


Human gut microbiota is:

- a complex community of 100 trillion archaeal and bacterial cells
- Composed by more than 1,000 bacterial species
- Composed by more than 90% from Firmicutes and Bacteroidetes.
- distinct and highly variable from person to person
- common among individuals (the core gut microbiota and the core microbiome)
- required for the correct functioning of the gut.

Il microbiota intestinale





Major Bacteria Phyla and Genera Predominating in Human Gut Microbiota

Phyla	Representative genera
Firmicutes (60-80%)	– Ruminococcus – Clostridium – Lactobacillus – Enterococcus
Bacteroidetes (20-30%)	– Bacteroides – *Prevotella*
Actinobacteria (< 10%)	– Bifidobacterium
Proteobacteria ((< 1%)	– *Escherichia* – *Enterobacteriaceae*

Fattori che influenzano la composizione del microbiota intestinale

ALLATTAMENTO

MODLITA' DI PARTO

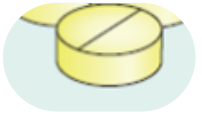
PROVENIENZA GEOGRAFICA

DIETA

ESERCIZIO FISICO

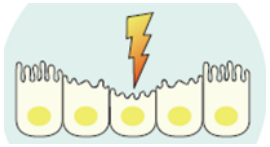
Composizione del microbiota intestinale umano

ANTIBIOTICI



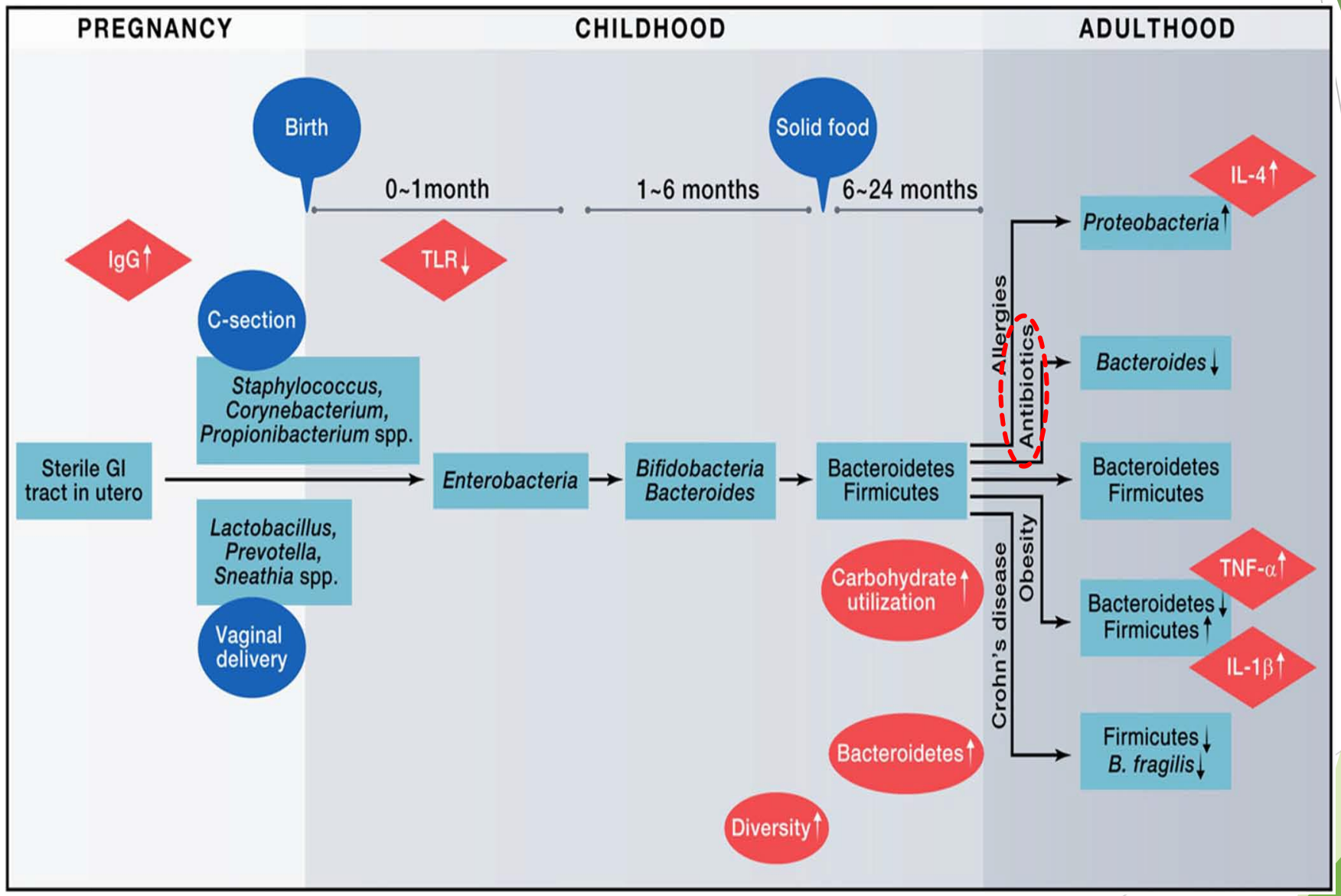
PRO/PREBIOTICI

AGING

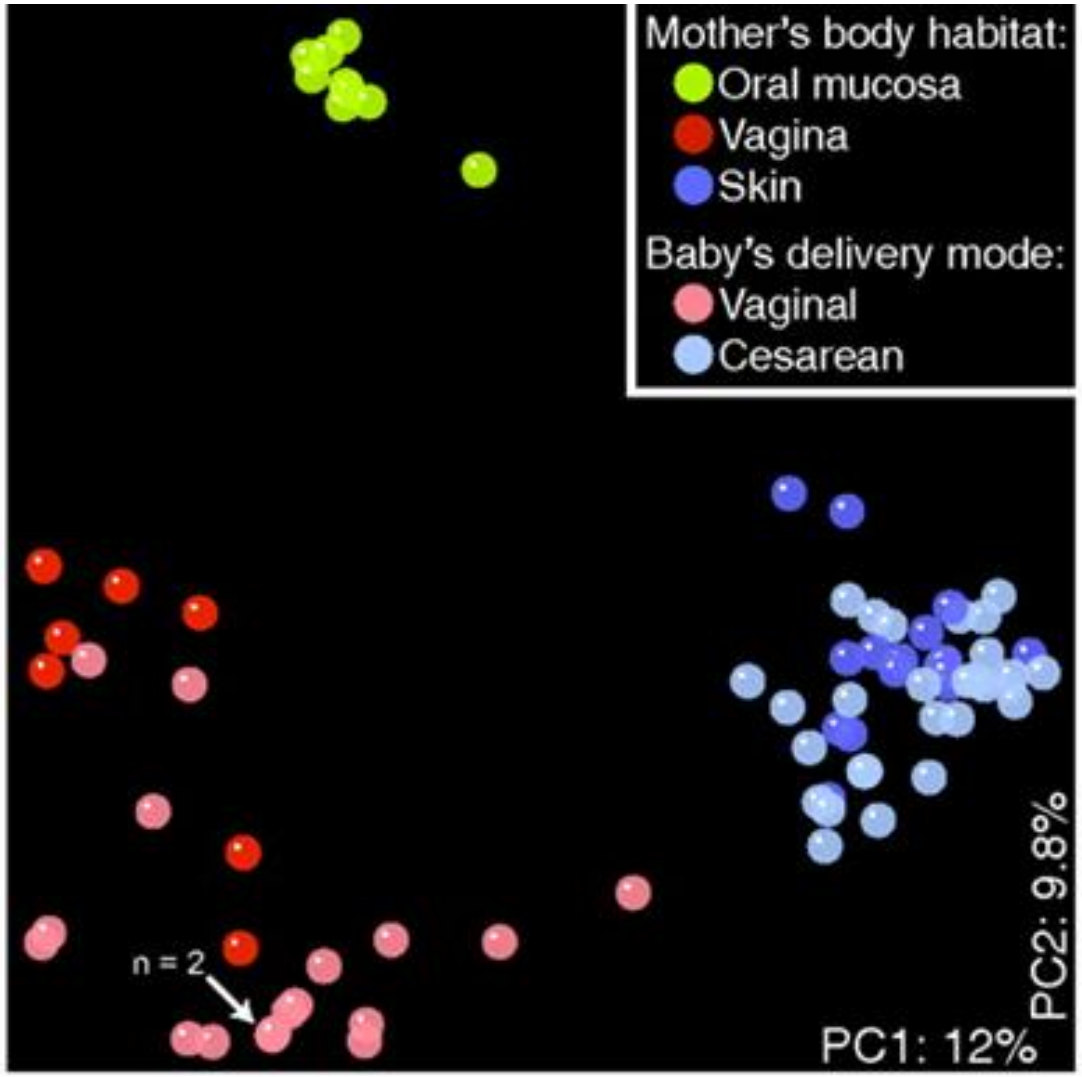


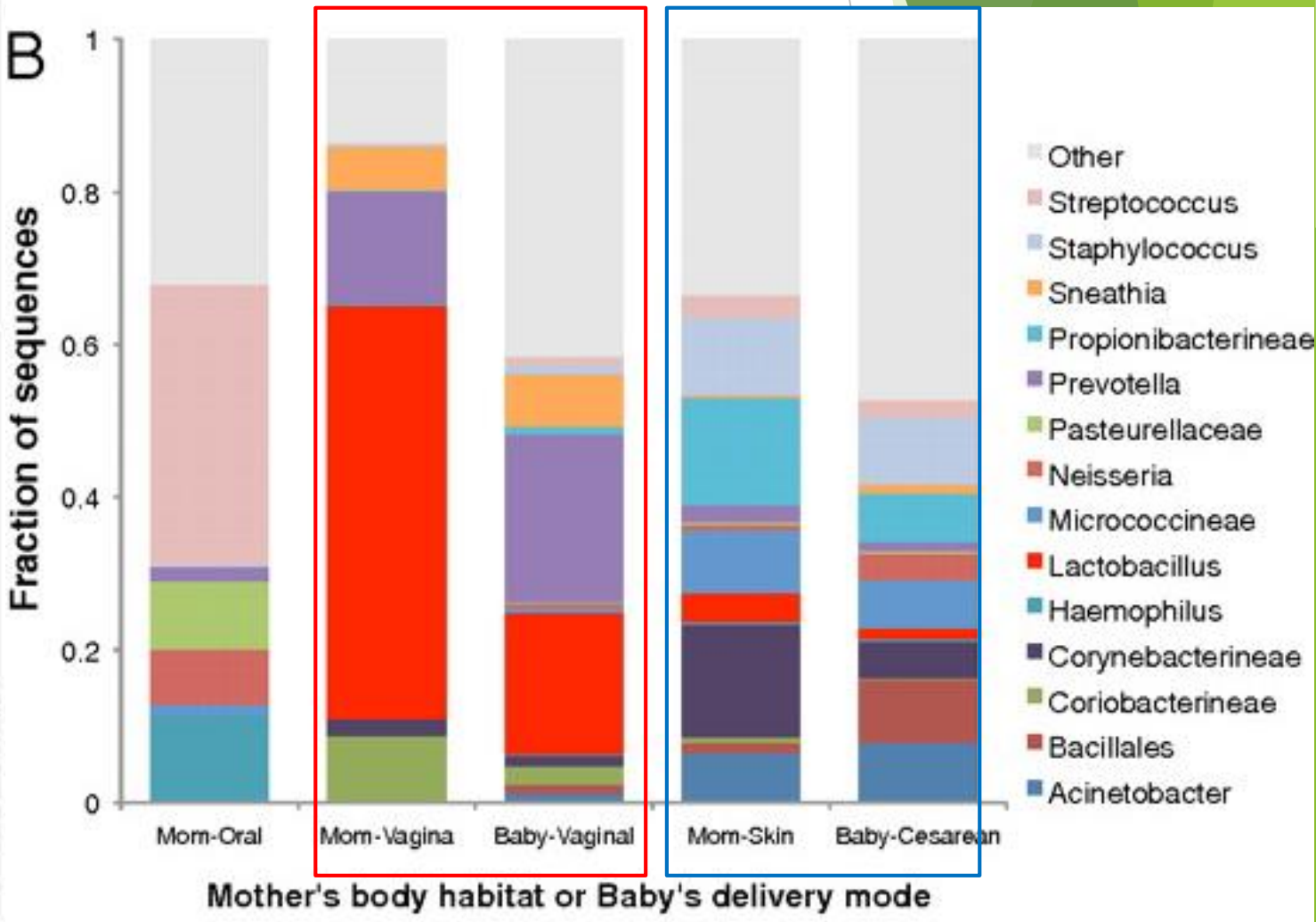
MALATTIE

Sviluppo del Microbiota

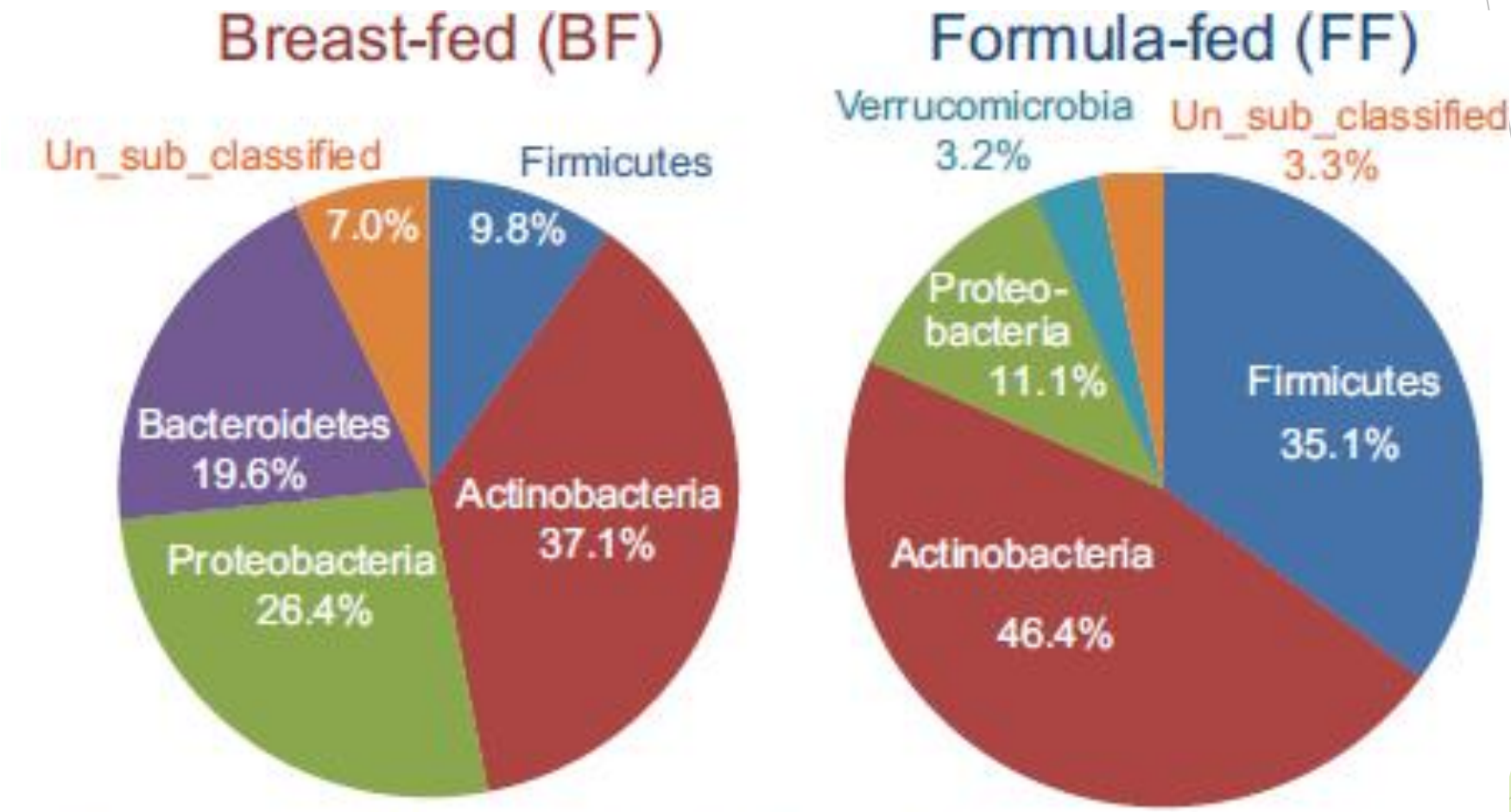


MODALITA' DI PARTO



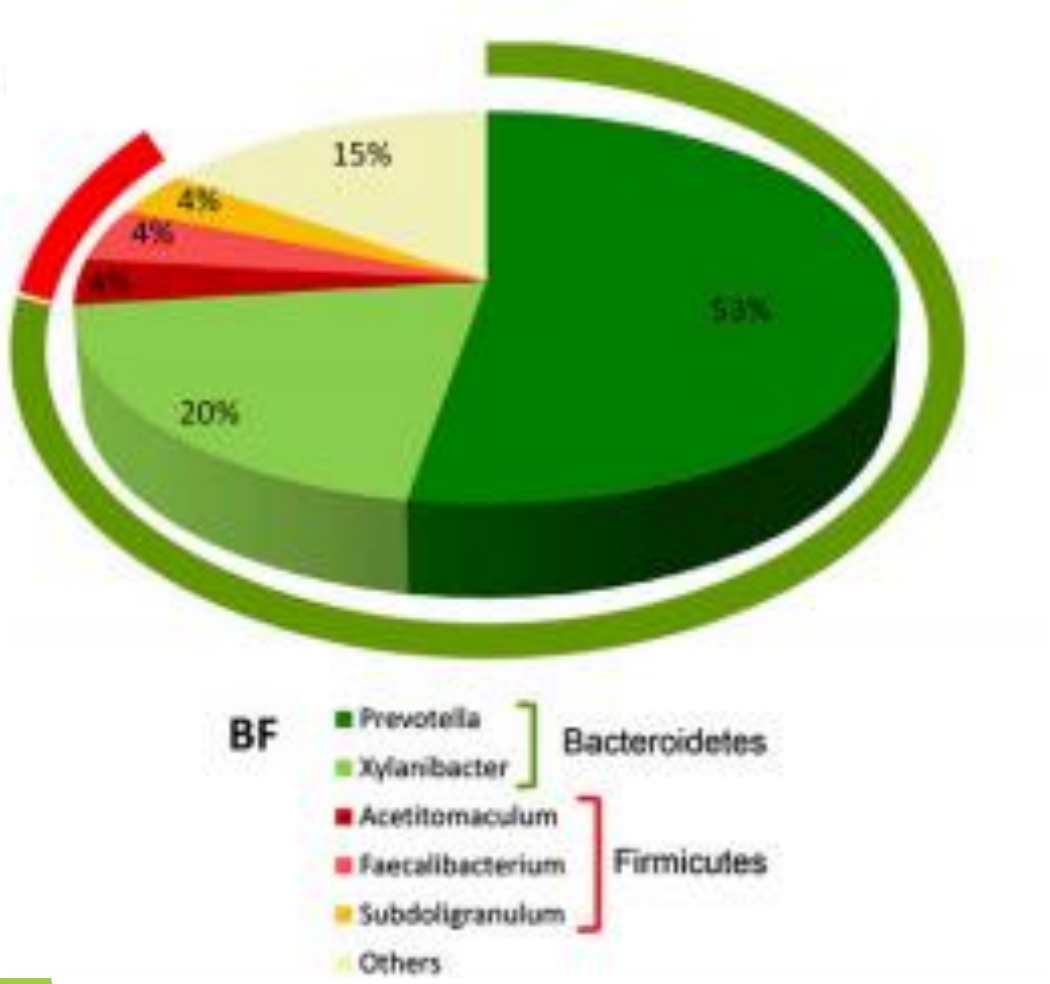


ALLATTAMENTO

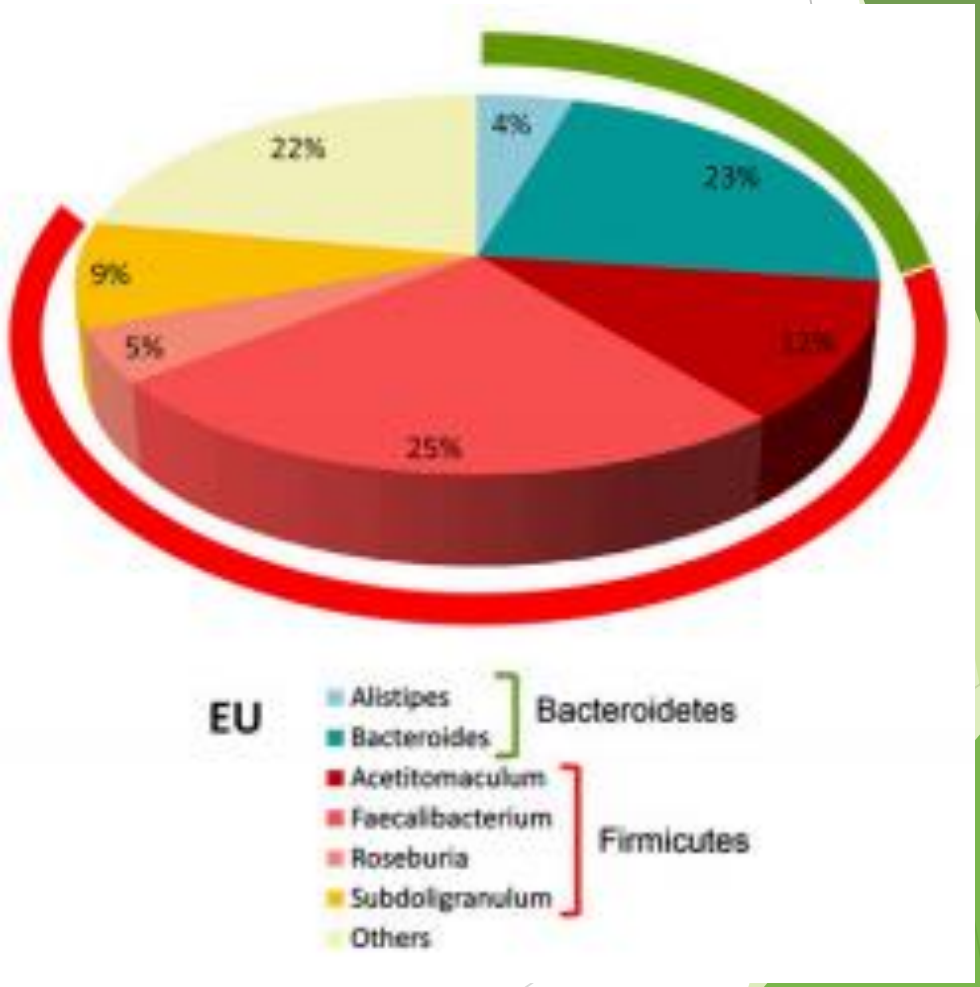


- BF had more total Proteobacteria and Bacteriodetes
- FF had more Firmicutes and no Bacteriodetes

ORIGINE GEOGRAFICA

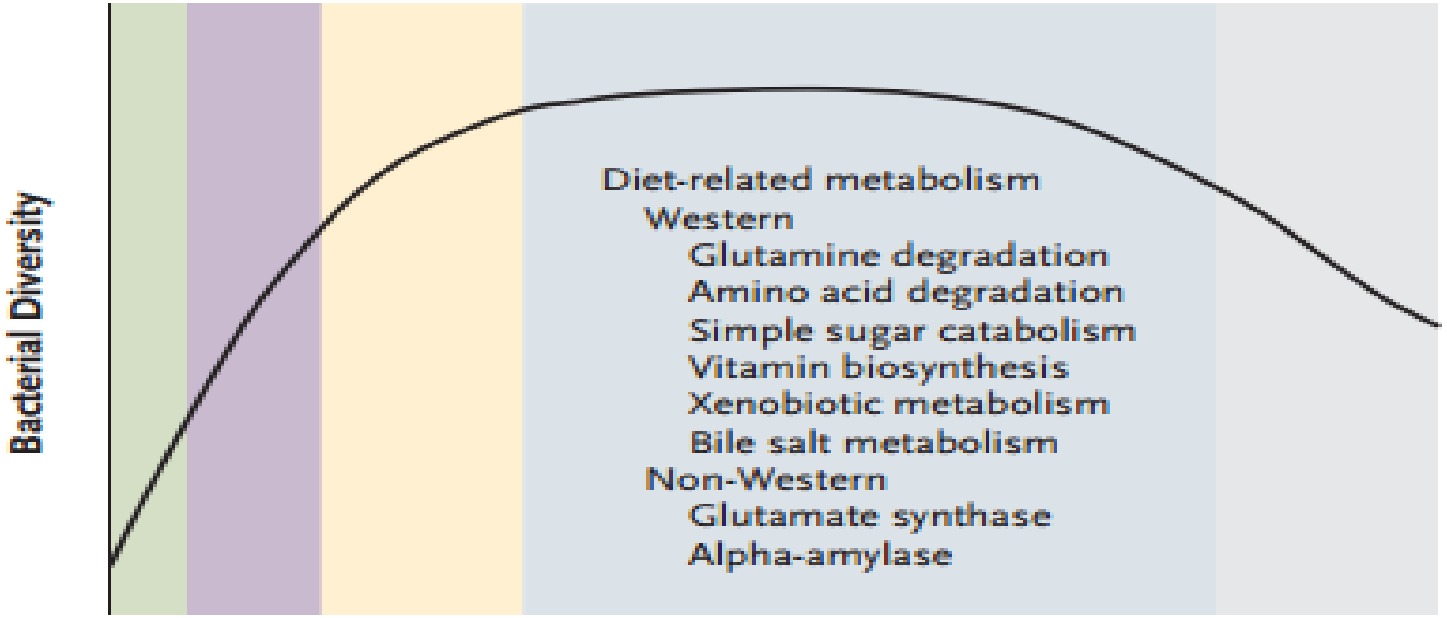


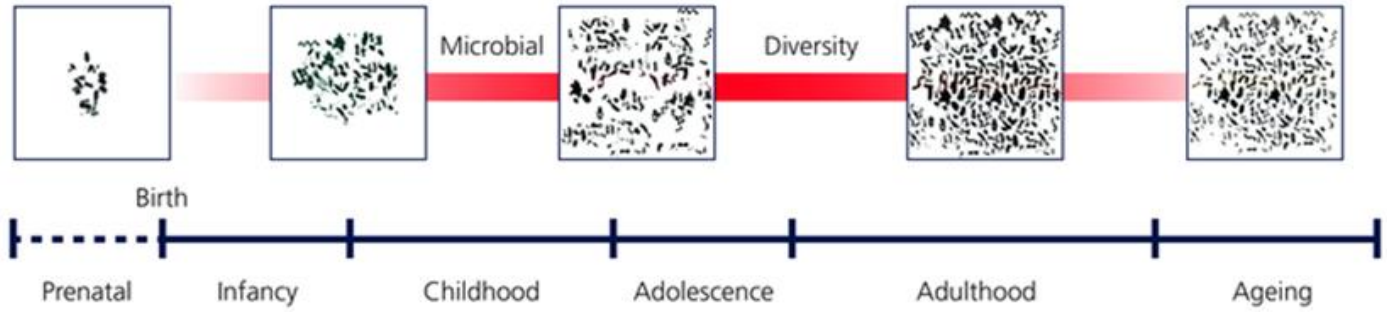
BURKINA FASO



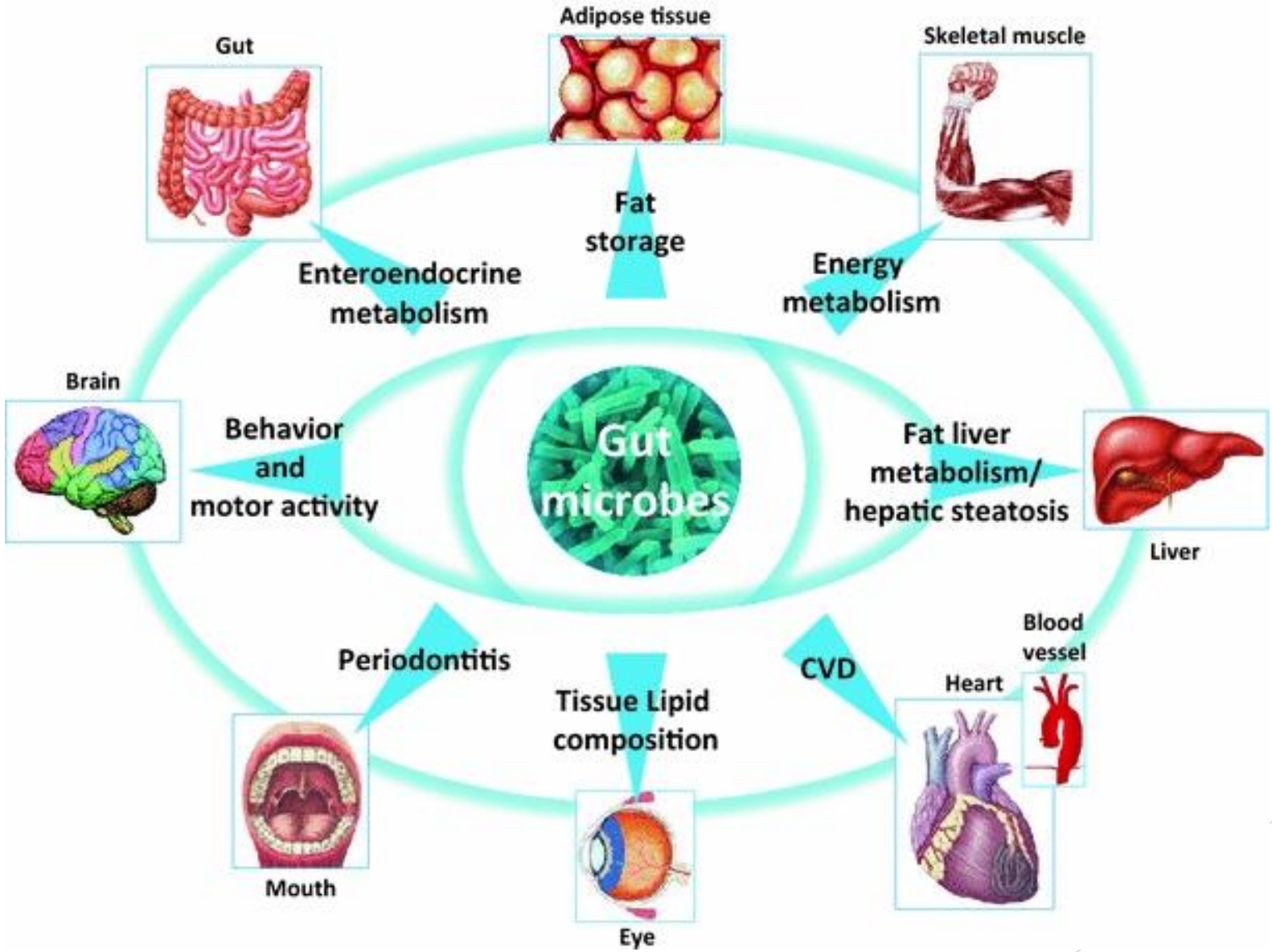
EUROPA

Il microbiota nelle diverse fasi della vita

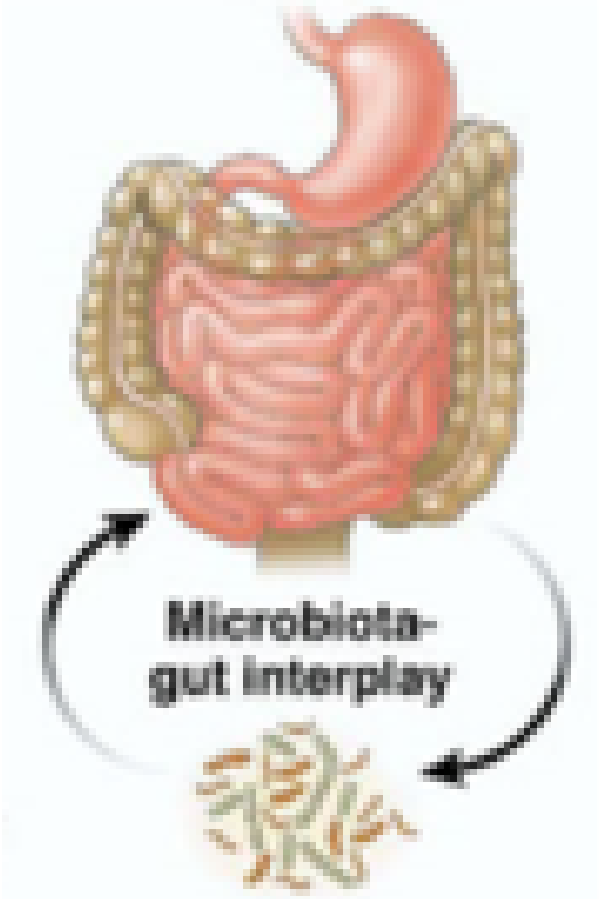




IL RUOLO DEL MICROBIOTA NELLA SALUTE



IL RUOLO DEL MICROBIOTA NELLA SALUTE



Metabolic

- Vitamines prouction
- Enterocytes differentiation
- Dietary carcinogenes digestion
- Non-digestible substrate fermantation
- SCFAs production

Antimicrobial secretion

Energy source

Sites and nutrients competition

Protective

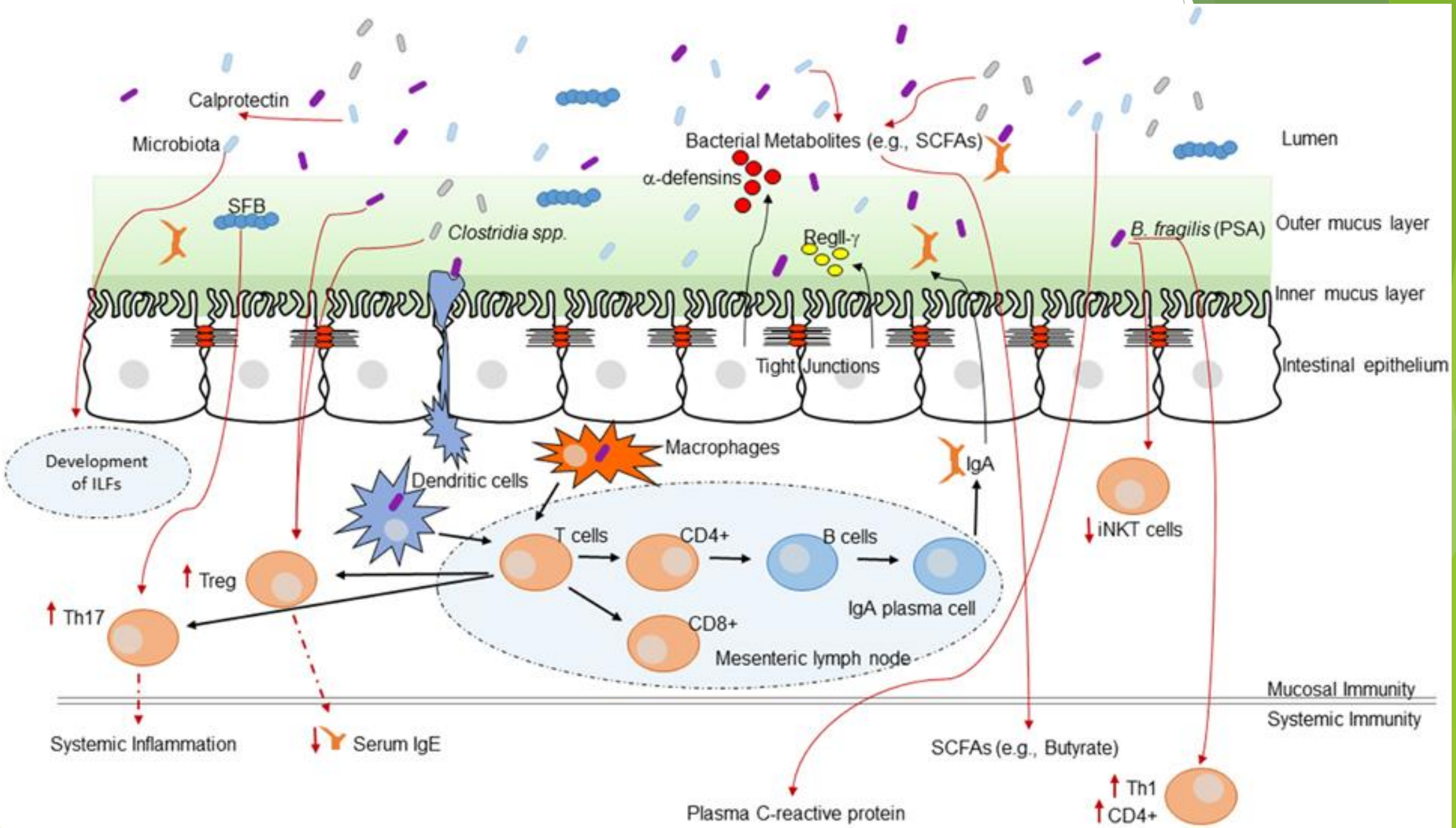
- Colonization resistance
- Innate and adaptative immunity
- Inflammatory cytokine oversite

Immune system and barrier function

Structural

- IgA stimulation
- Intestinal villi and crypts stimulation
- Tight junction
- Mucous layer production

IL RUOLO DEL MICROBIOTA NELL'IMMUNITA'

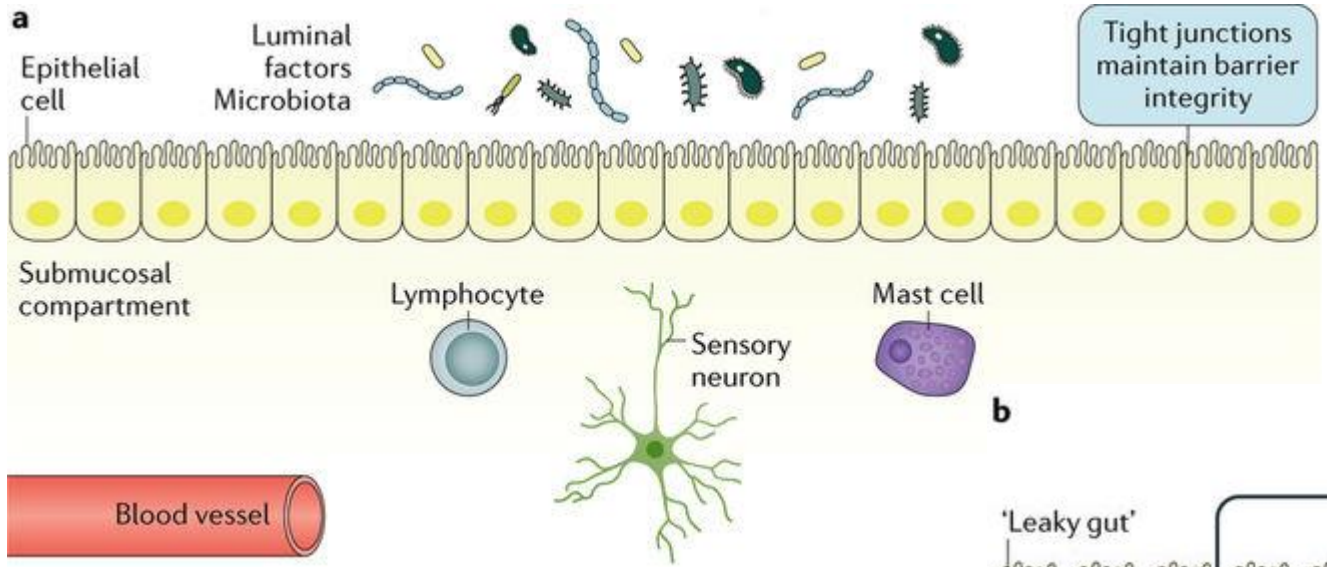


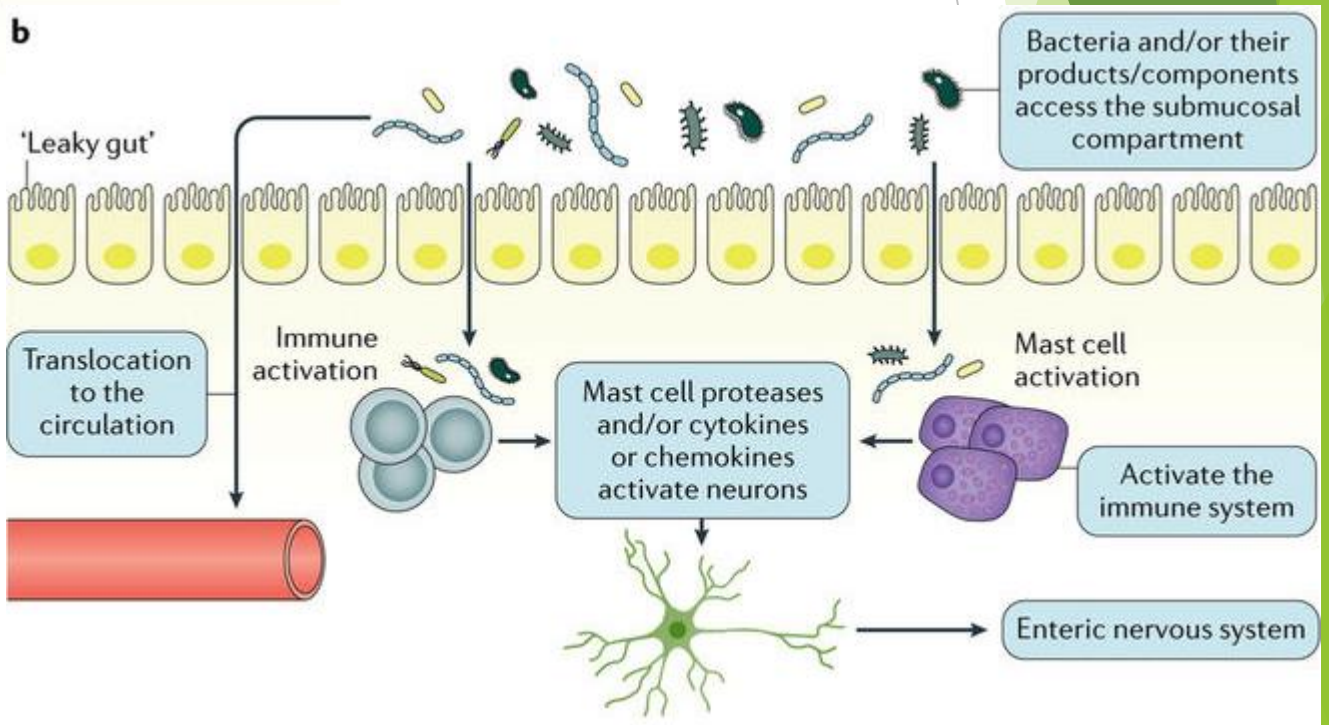
Protective function (barrier effect)

- Compete and adhere to the attachment sites in the brush border of intestinal epithelial.
- Compete for available nutrients.
- Produce antimicrobial (bacteriocins).

All of this will prevent attachment and subsequent entry of pathogenic bacteria into the epithelial cells

The 'leaky gut' hypothesis



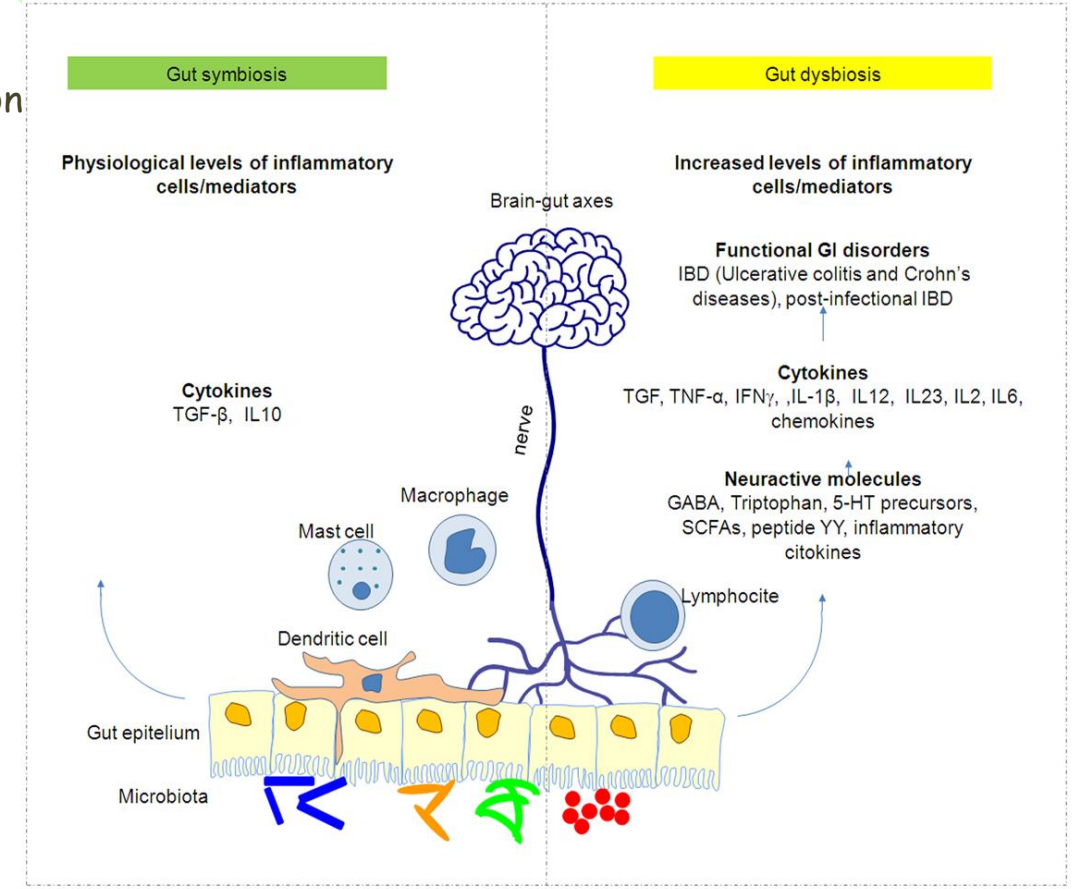


Gut microbiota symbiosis and dysbiosis

HEALTH ← HOMEOSTASIS

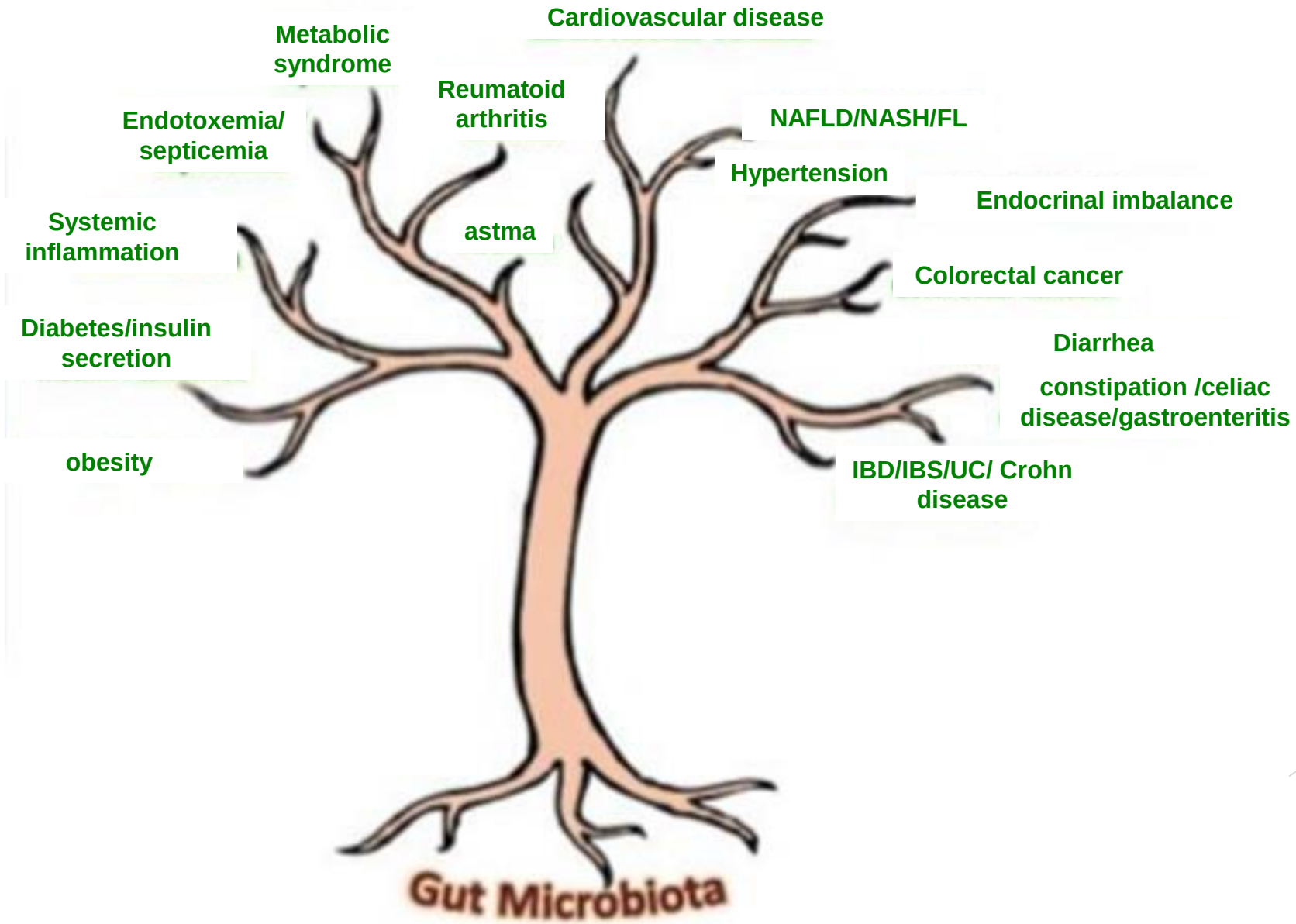
DYSFUNCTION → DISEASE

- Vitamin production
- Resistance to infection
- Immune system stimulation
- Organ vitality
- Healthy ageing



- Cystic fibrosis
- Fatty Liver Diseases (NAFLD and NASH)
- Juvenile idiopathic arthritis
- Obesity
- Early Inflammatory bowel diseases (IBD)
- MDR infection
- Allergy
- Dermatitis

IL RUOLO DEL MICROBIOTA NELLE MALATTIE



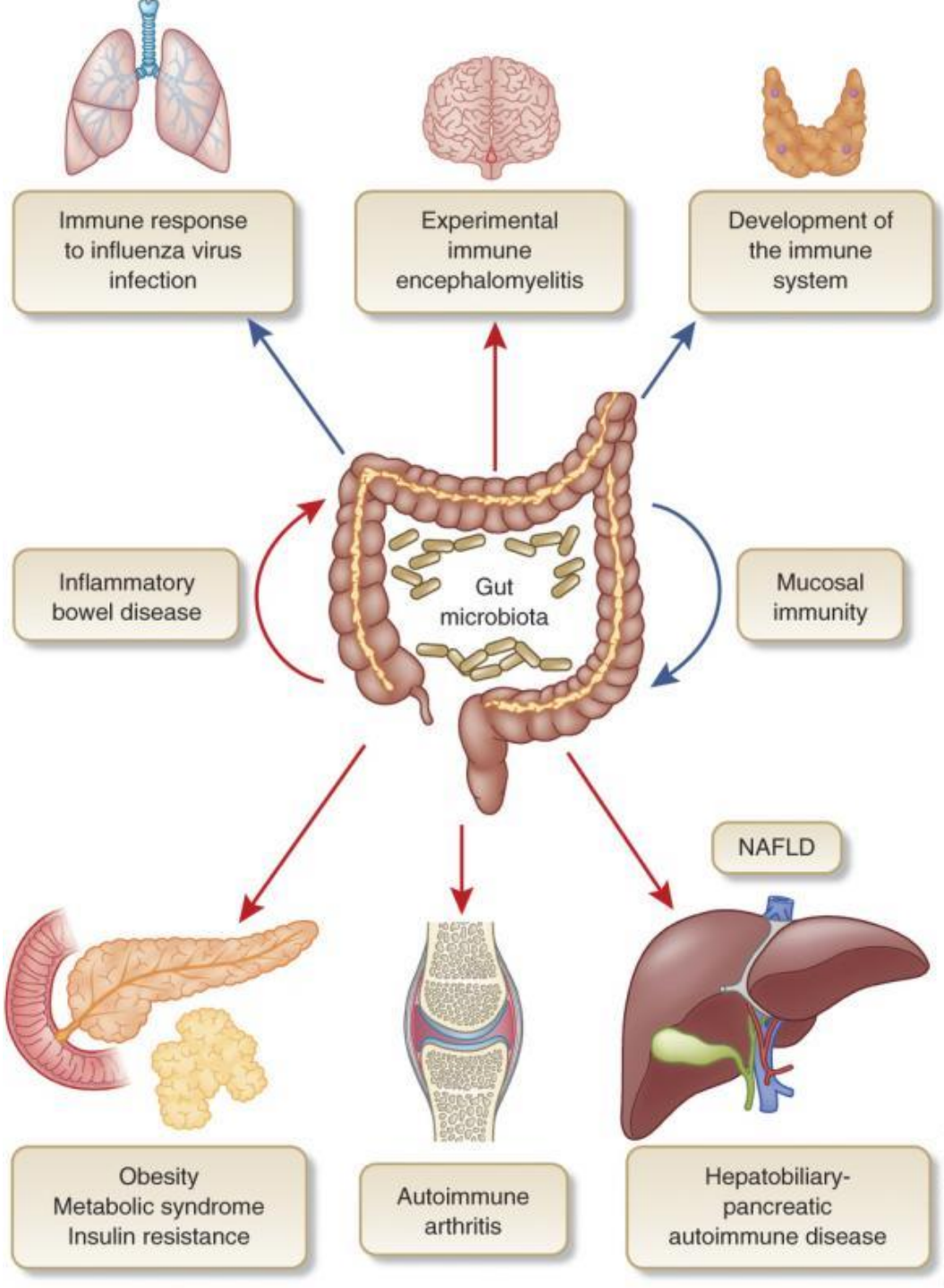
DISBIOSI

- Fattori genetici che possono alterare la barriera intestinale
- Crescita smisurata di batteri patogeni
- Traslocazione di batteri o prodotti batterici
- Attivazione immunitaria produzione di citochine pro-infiammatorie
- Infiammazione cronica che porta alla distruzione dei tessuti
- Leaky-gut

PATOLOGIE CORRELATE AL MICROBIOTA

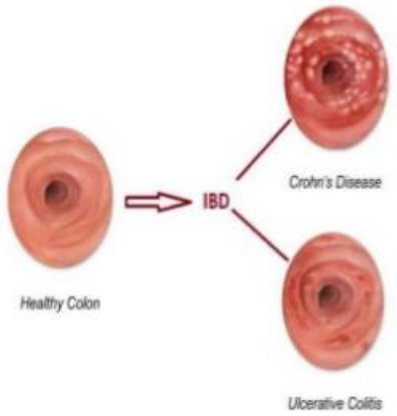
- **Diseases of the GUT**
  - Malabsorption syndrome
  - Malignancies: Colorectal cancer
  - Inflammatory Bowl disease (IBD)
  - Irritable Bowl syndrome
  - Diarrheal diseases
  - Clostridium Difficile Infection (CDI)

- **Non-mucosal diseases**
  - Obesity and metabolic syndrome
  - Malignancies: liver cancer, breast cancer
  - Complications of liver cirrhosis
  - Allergic conditions
  - Autoimmune disorders (T1DM, arthritis etc)
  - Abnormalities of the gut-brain axis- Autism an other neurological disorders
  - Obesity and other metabolic disorders
  - Chronic fatigue syndrome
  - Periodontal diseases



Inflammatory Bowl Disease (IBD)

A group of inflammatory and autoimmune conditions that affect the colon and small intestine, typically resulting in severe abdominal pain, weight loss, vomiting and diarrhea.



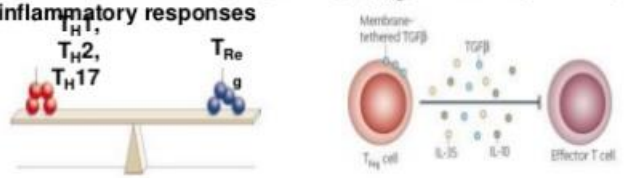
- Affects all layers of the bowel wall
- Granuloma formation in up to 60% of patients

- Affects superficial mucosal layers

IBD is driven by T cells

mucosal homeostasis

→ cytokine production by regulatory (T_{Reg}) T cells supresses pro-inflammatory responses



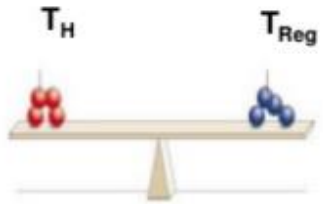
mucosal inflammation

→ increased production of pro-inflammatory cytokines by T helper (T_H) cells

TNF, IFNγ, IL-17

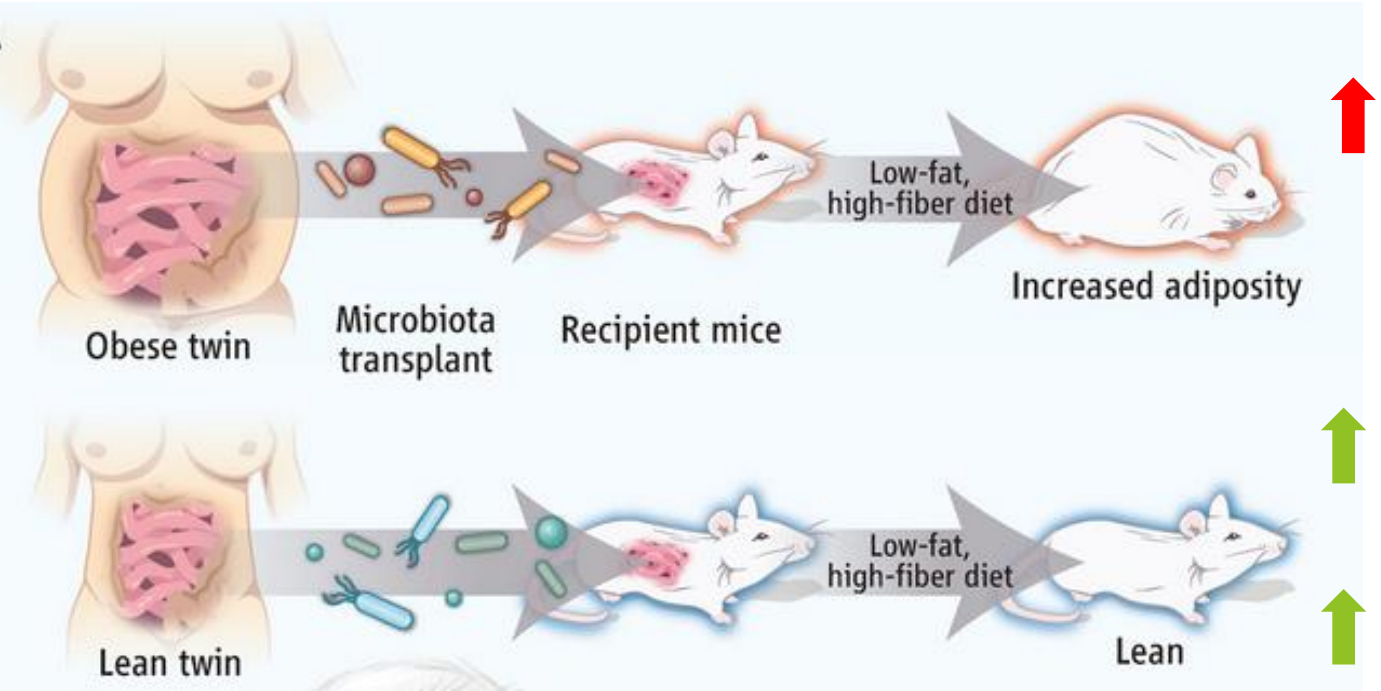
← T_{Reg} transfer can prevent the induction of experimental colitis

Involvement of the microbiota in regulating the balance between T_H and T_{Reg} cell subsets in the gut



→ Intestinal bacteria direct the differentiation of both pro- and anti-inflammatory T cell populations and may therefore play a crucial role in IBD

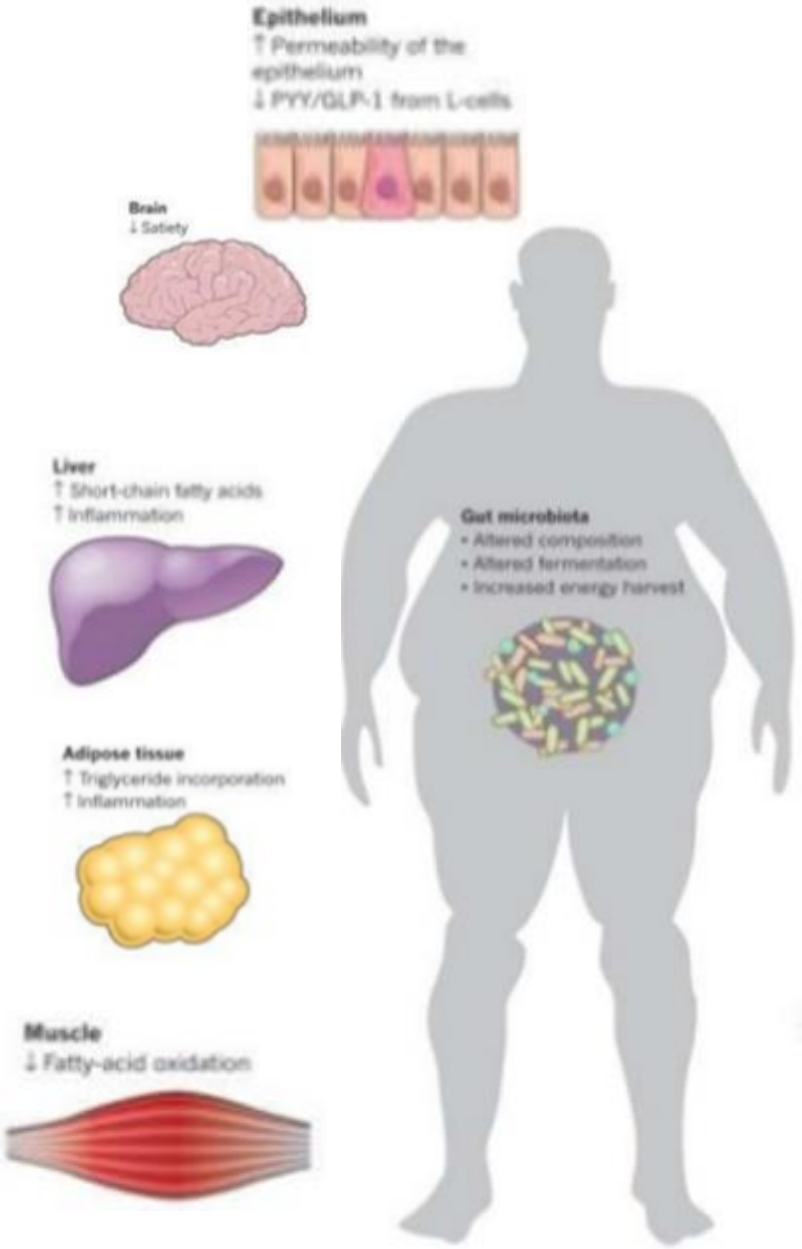
Gut microbiota and obesity: what is the link?



BCFA metabolism

SCFA fermentation

Bile acid microbial transformation



Oltre i batteri…..

Characteristic	Bacteria	Viruses	Eukaryotic microbes
Genome size	0.5–10 megabases	1–1,000 kilobases	10–50 megabases
Number of taxa in the human microbiome	At least thousands	Unknown, but could be as many as bacteria	Unknown, but may be fewer than bacteria
Relative abundances	Highly variable	Highly variable	Unknown
Targeted detection methods	Sequencing of genes such as 5S and 16S rRNA	No universal method for genes, but virus-specific polymerase chain reaction assays for some	Sequencing of 18S rRNA gene Spacer region in rRNA
Shotgun approach to analyses	Alignment to reference genomes or database comparison	Database comparison	Alignment to reference genomes or database comparison
Subspecies or strain diversity	Modest sequence variation Horizontal gene transfer also contributes	High sequence variation	Unknown

Disegno dello studio

Ipotesi dello studio

Studio longitudinale

cambiamenti nel microbiota (trattamenti farmacologici, stati di malattia)

Studio trasversale (cross sectional)

correlazione microbiota fenotipi clinici (relazione tra microbiota e patologie)

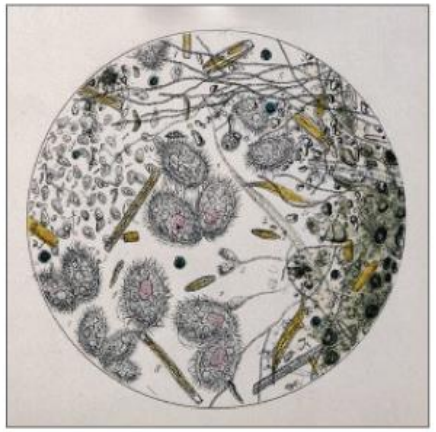
Scelta del campione

Table 1 | **Advantages and disadvantages of sample types for gut microbiome analysis**

Sample	Advantages	Disadvantages
Faecal sample	Noninvasive; no bleeding or discomfort; no bowel cleansing; easier to sample frequently	A proxy for the gut microbiome; might contain dead bacteria and/or bacteria from unspecified gastrointestinal tract compartments; less controlled sampling variables
Luminal brush	Captures host–microbe interactions; increased mucosal coverage; no bleeding; greater proportion of bacterial to host DNA than biopsies	Requires endoscopy; less biomass for host studies; affected by bowel cleansing
Rectal swab	No bleeding; greater proportion of bacterial to host DNA than biopsies; no bowel cleansing; can be administered at home; easier to sample frequently	No visual aid to pinpoint areas of interest; limited biomass for host studies; more discomfort than stool sampling; potential contamination with skin bacteria
Colonic lavage	Provides more DNA than biopsy samples; no bleeding	A proxy for the gut microbiome; requires endoscopy; affected by bowel cleansing
Pinch biopsy	Captures host–microbe interactions; can target exact areas of interest	Requires endoscopy; disrupts epithelium; affected by bowel cleansing
Sub-mucosal biopsy	Captures host–microbe interactions and bacterial translocations through epithelial layers; can target exact areas of interest	Requires endoscopy; disrupts epithelium; requires extensive sample processing; affected by bowel cleansing

Metodi di indagine del microbiota

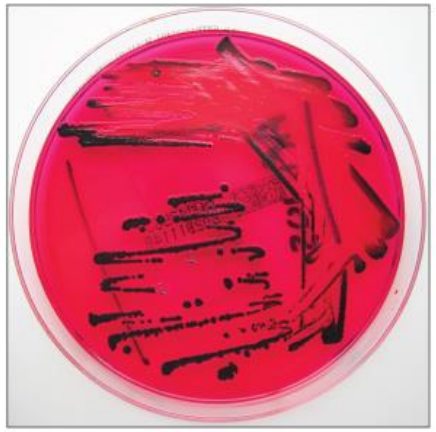
circa 1600:
Microscope invented

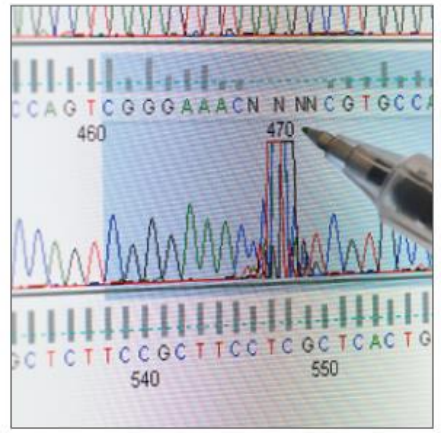


mid-1600s:
First microbes described

1800s:
Connection made between microbes and disease

1800s – Present:
Culture, staining, and microscopy used to study microbes that can be cultured

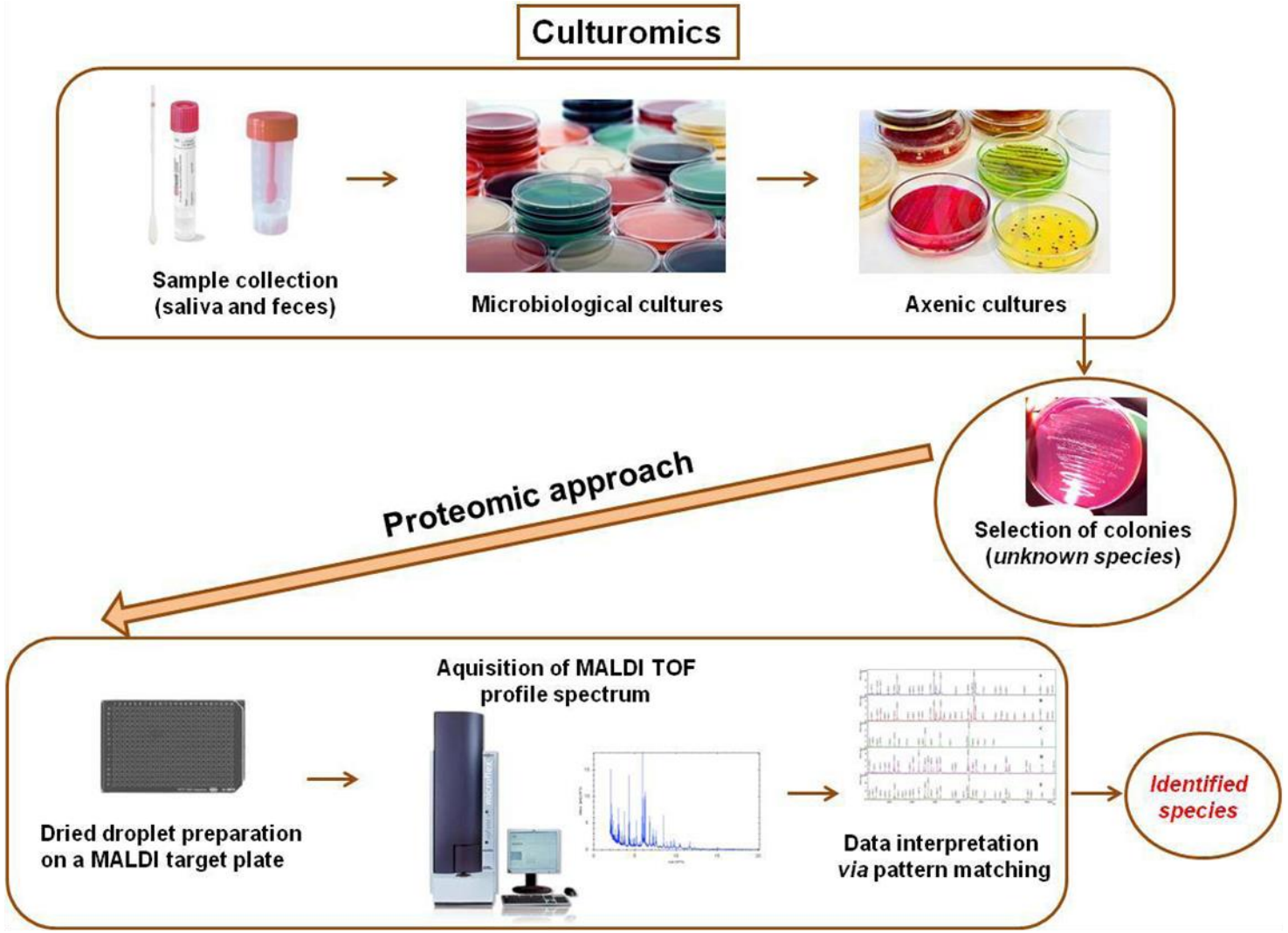




1990s:
DNA sequencing becomes available, allowing study of microbes that cannot be cultured

Metodi di indagine del microbiota

tecniche standard di microbiologia



99% of microbial species cannot currently be cultivated: Culturing: a few hundreds species per gram

Del Chierico, Gnani, Vernocchi et al., 2014. Meta-omic platforms to assist in the understanding of NAFLD gut microbiota alterations: tools and applications. ***Int J Mol Sci***. 2014 Jan 7;15(1):684-711

Metodi di indagine del microbiota

Tecnologie «OMICHE»

Quali microrganismi sono presenti?

ANALISI FILOGENETICA
Target: 16S rRNA

Qual è il potenziale genetico del microbiota?

ANALISI METAGENOMICA
Target: DNA

Quali attività stanno svolgendo?

ANALISI METAPROTEOMICA
Target: Proteine

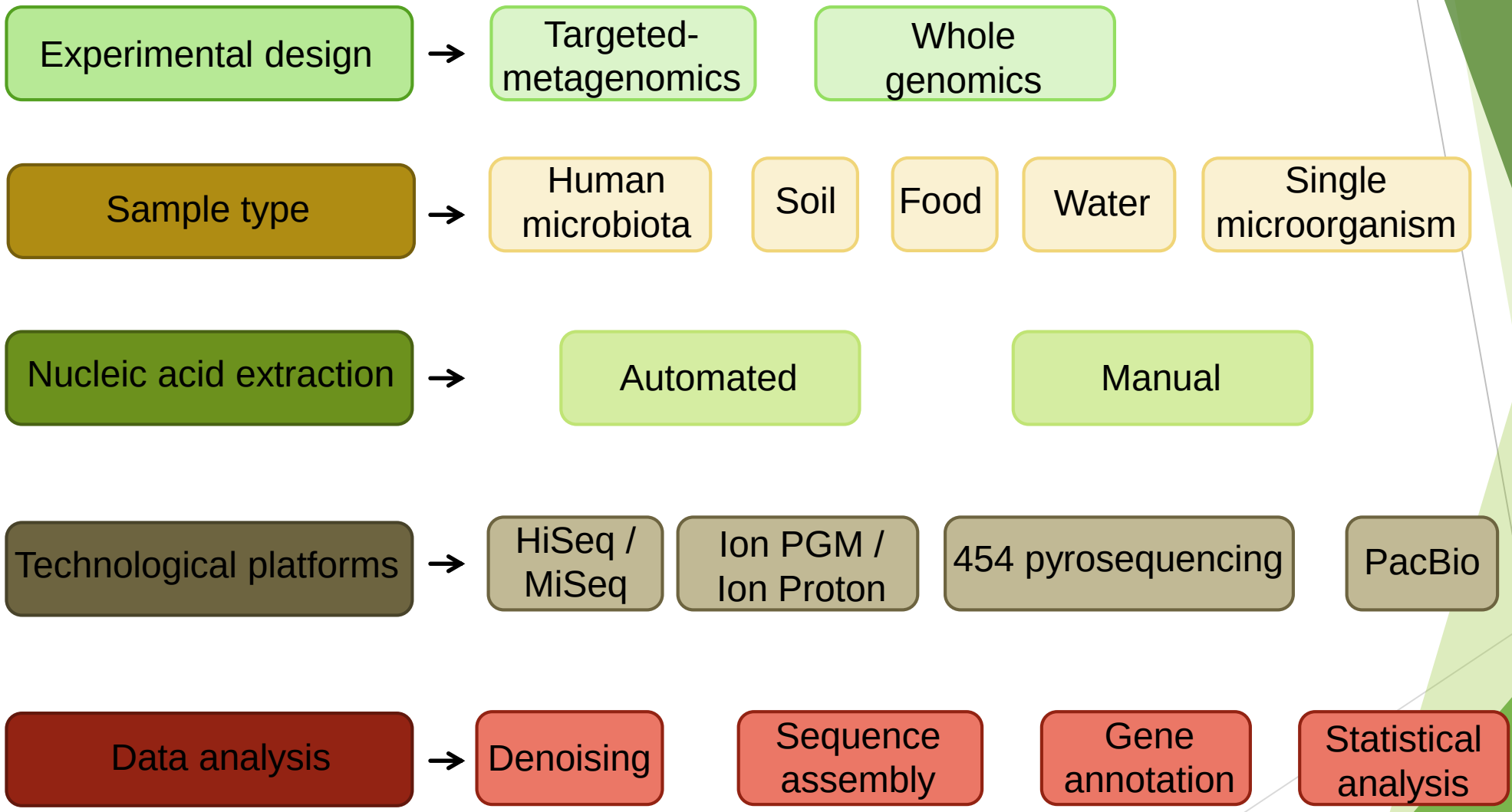
ANALISI METABOLOMICA
Target: Metaboliti

NGS sequencing

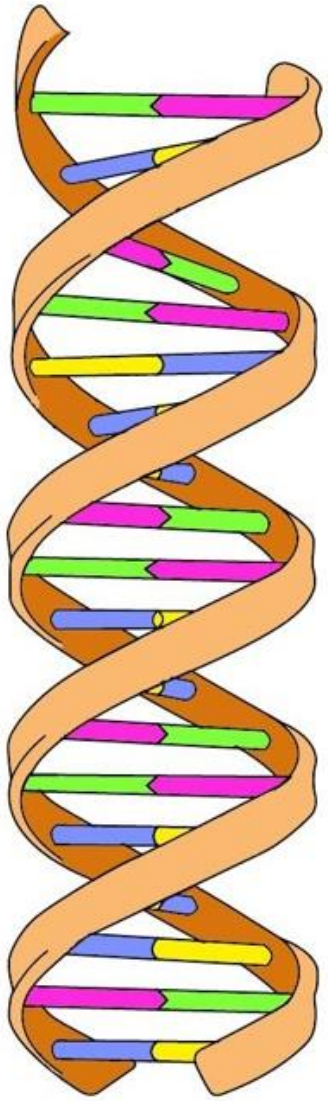
Culture-indipendent DNA-based methods

Platform	Method	Characteristics	16S rRNA	Shotgun	Comments
Established					
Sanger-based or capillary-based instrument	Fluorescent, dideoxy terminator	750-base reads High accuracy	Full length sequenced with 2–3 reads	Long reads help with database comparisons	Most costly method Relatively low throughput, so low coverage of 16S or shotgun
Roche–454	Pyrosequencing light emission	400-base reads	Up to 3 variable regions per read	Long reads help with database comparisons	Cost limits shotgun coverage but 16S coverage is good
Illumina	Fluorescent, stepwise sequencing	100–150- base reads	Only 1 variable region per read	Short reads do not seem to limit analysis	Very high coverage owing to high instrument output and very low cost
Not yet widely used					
Ion Torrent	Proton detection	More than 200-base reads	Like other NGS	Like Illumina	Expect high coverage, but longer reads than Illumina
PacBio	Fluorescent, single-molecule sequencing	Up to 10-kilobase reads Low accuracy	Accuracy an issue for correct taxon identification	Long reads could help assembly	Attractive for long reads, but lower accuracy limits applications
Oxford Nanopore*	Electronic signal as DNA passes through pore Single-molecule sequencing	Long reads	Unknown	Long reads could help assembly	Not yet available

Next generation sequencing



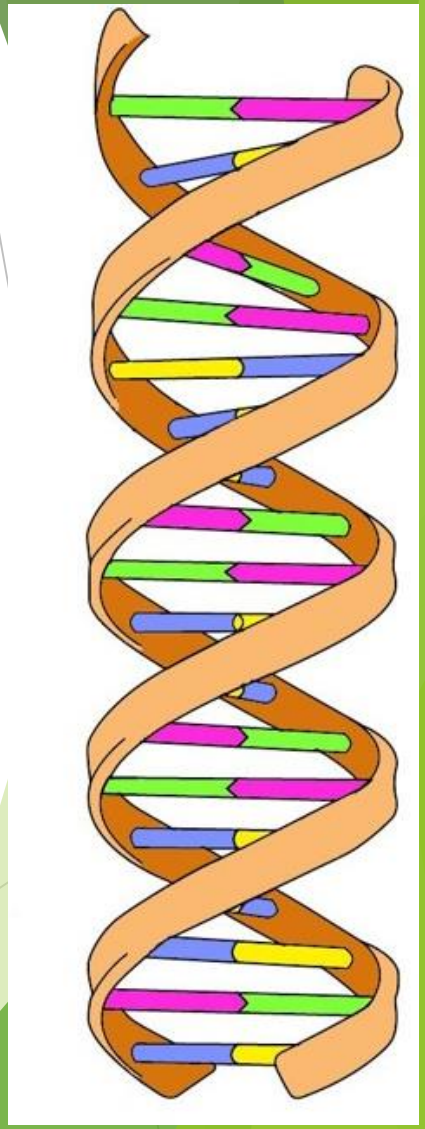
Il DNA è un ottimo strumento per la tassonomia



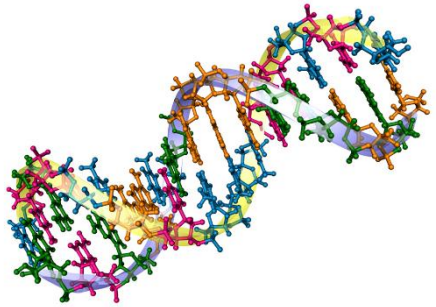
Le sequenze di DNA hanno numerosi vantaggi rispetto ai caratteri morfologici:

- Marcatori possono essere determinati in modo non ambiguo (variazioni di sequenza)

- Il DNA codificante per l'RNA ribosomale batterico è il miglior marcatore batterico

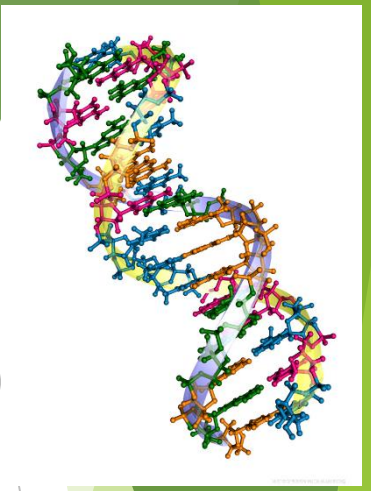


Estrazione del DNA

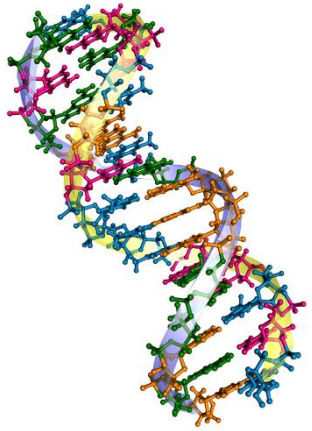


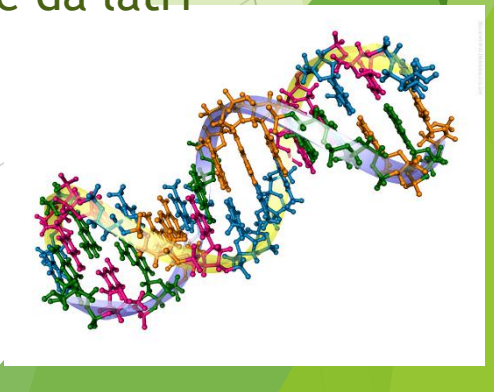
Requisiti
fondamentali:

- quantità sufficiente
- alta qualità
- contenere una rappresentazione fedele della comunità microbica presente nel campione.



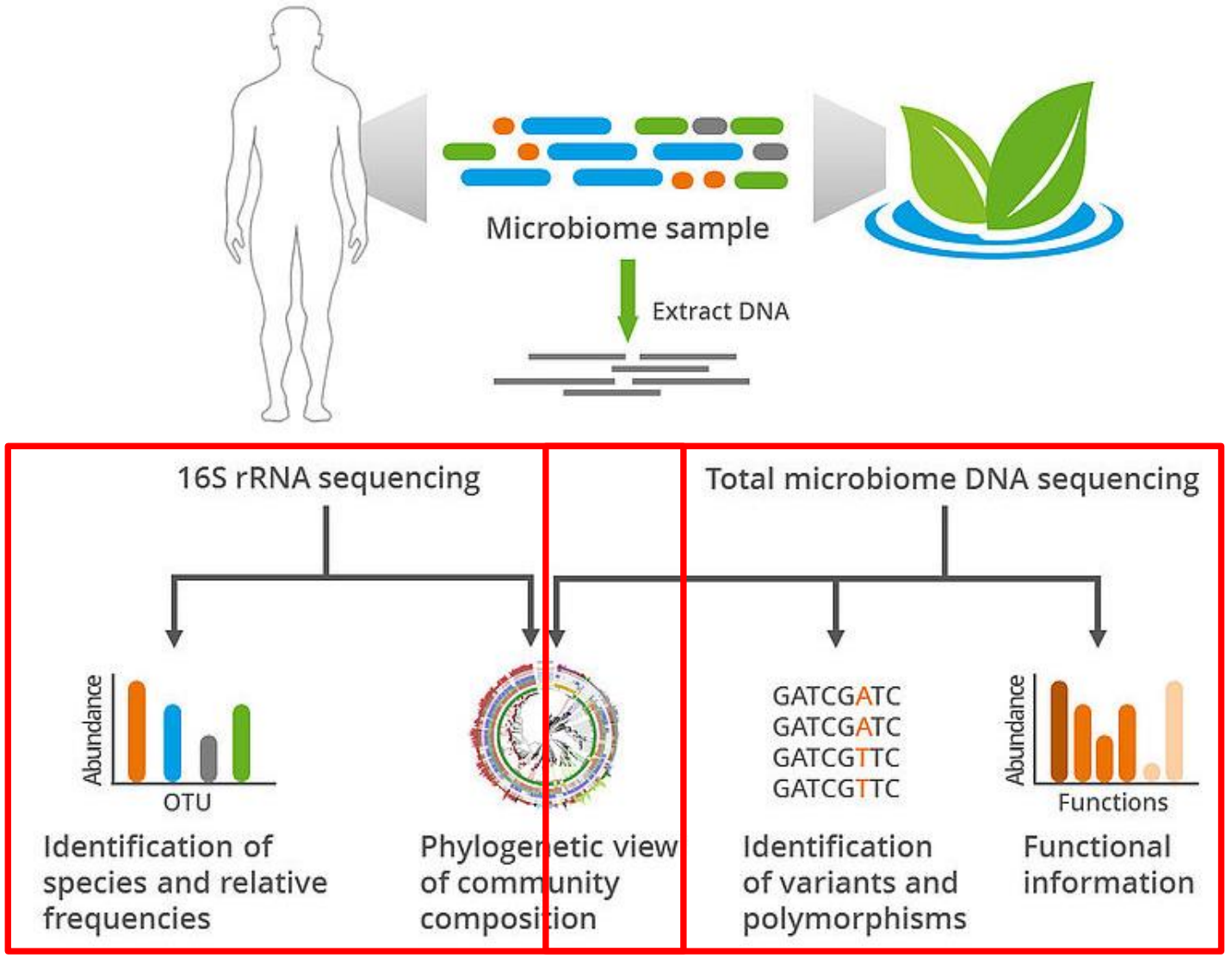
L’estrazione del DNA da campione fecale può risultare una procedura complessa a causa dell’elevata presenza
di DNA proveniente da altre matrici come cellule umane e cibo, e da latri contaminanti come i metaboliti cellulari.





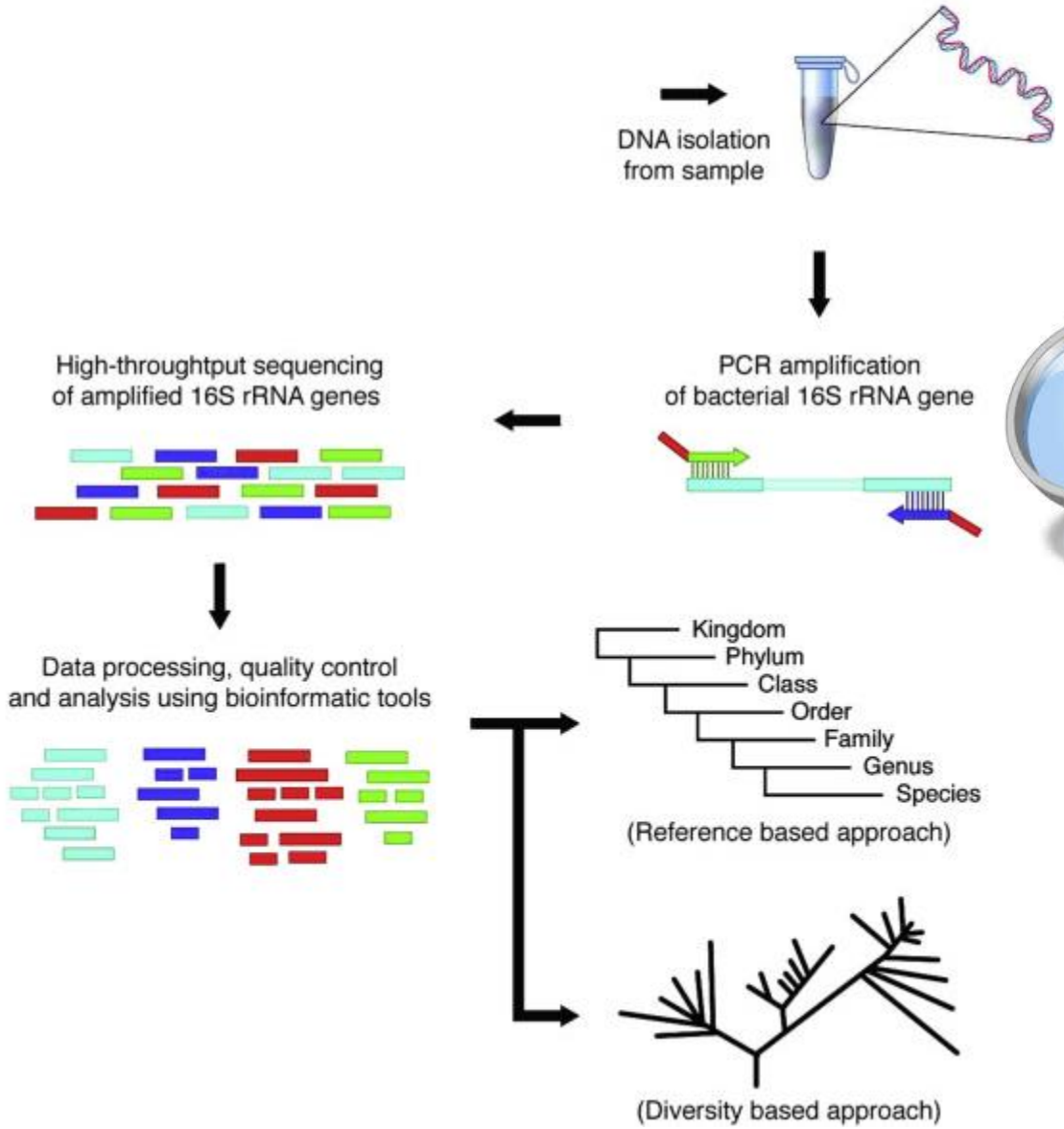
Metagenomica

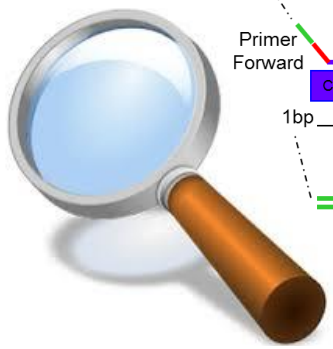
Analisi delle comunità microbiche mediante metodi indipendenti dalla cultura microbica

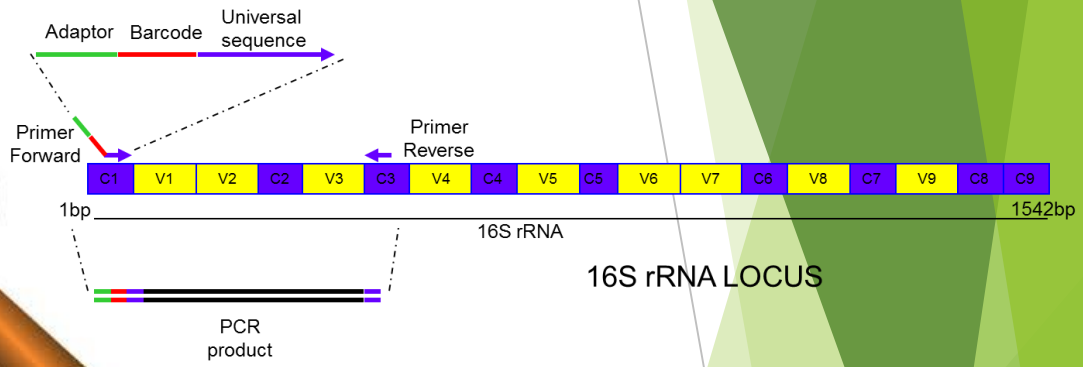


OTU = Operational Taxonomic Unit, a group of very similar 16S sequences

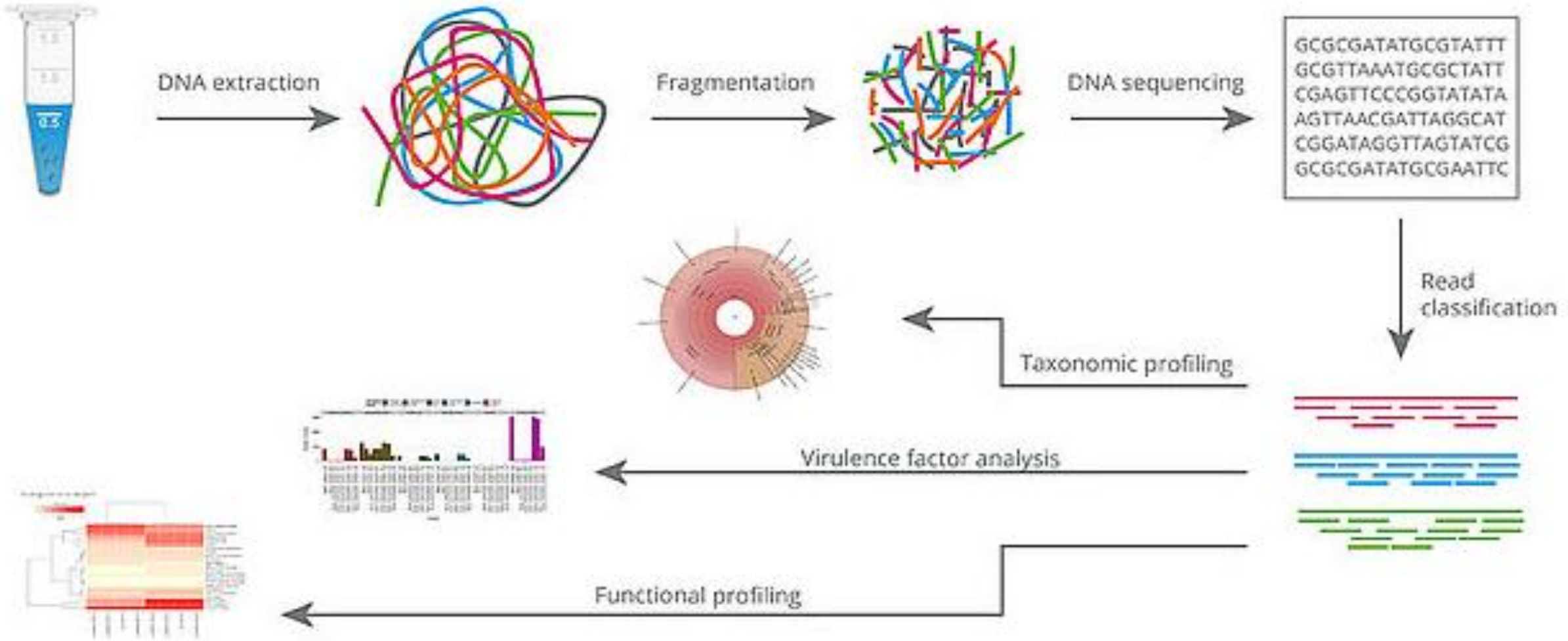
Metagenomica 16S-rRNA-based







Metagenomica Shotgun



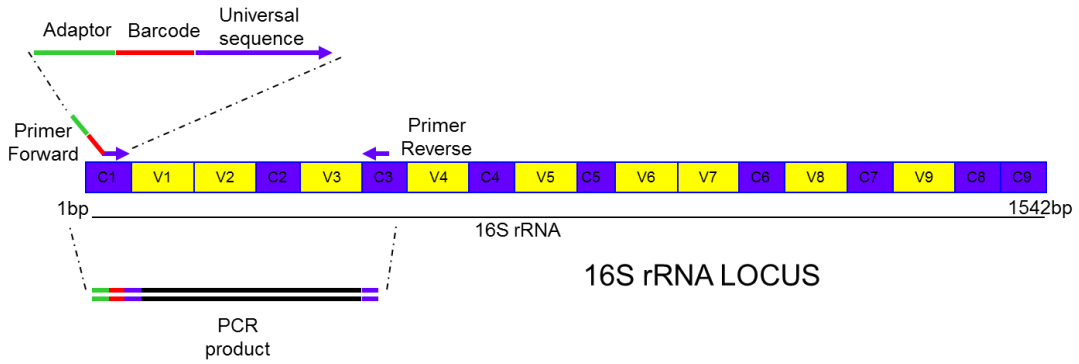
Metagenomica 16S-rRNA-based

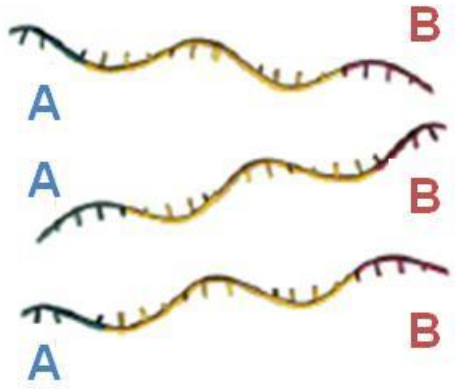
- Pros
  - Well established
  - Sequencing costs are relatively cheap (~50,000 reads/sample)
  - Only amplifies what you want (no host contamination)
- Cons
  - Primer choice can bias results towards certain organisms
  - Usually not enough resolution to identify to the strain level
  - Different primers are needed for archaea & eukaryotes (18S)
  - Doesn't identify viruses

Metagenomica Shotgun

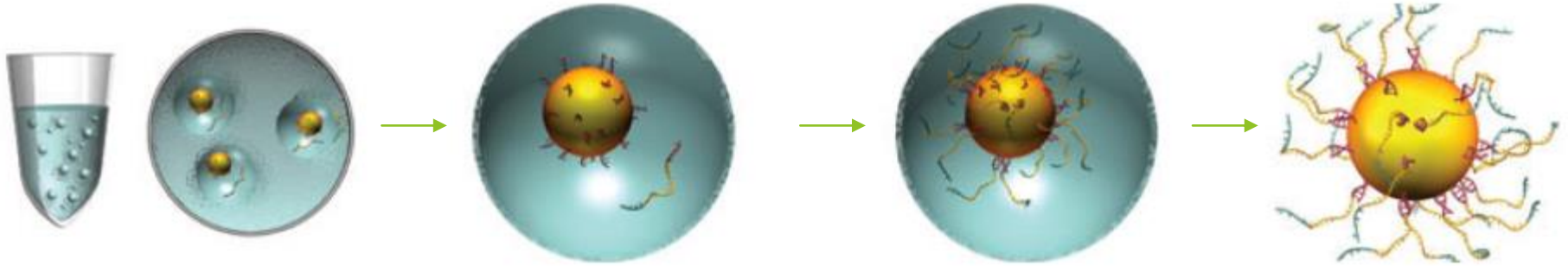
- Pros
  - No primer bias
  - Can identify all microbes (euks, viruses, etc.)
  - Provides functional information ("What are they doing?")
- Cons
  - More expensive (millions of sequences needed)
  - Host/site contamination can be significant
  - May not be able to sequence "rare" microbes
  - Complex bioinformatics

454-PYROSEQUENCING



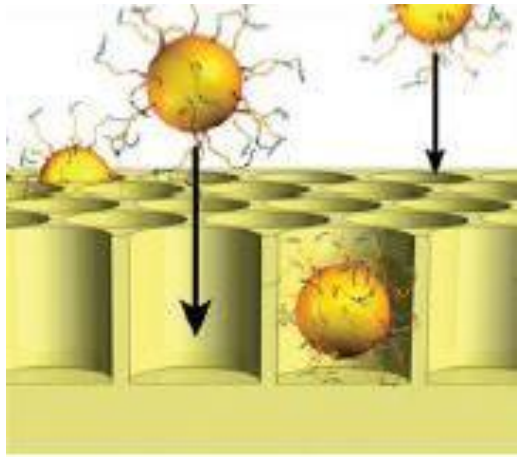


Single strand fragments

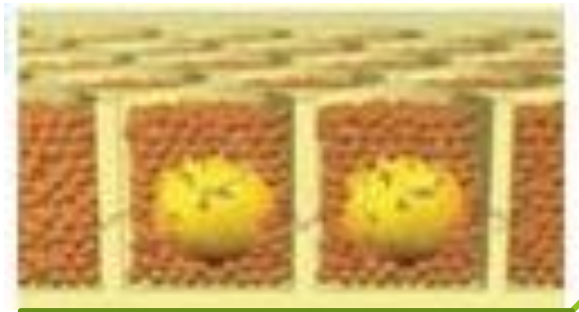


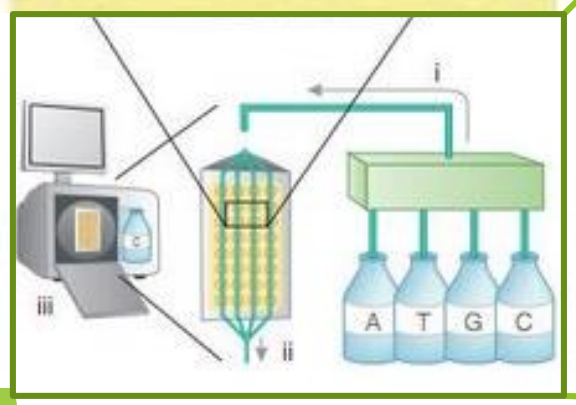
Emulsion → Bead ligation → Clonal amplification → DNA positive beads

454-PYROSEQUENCING

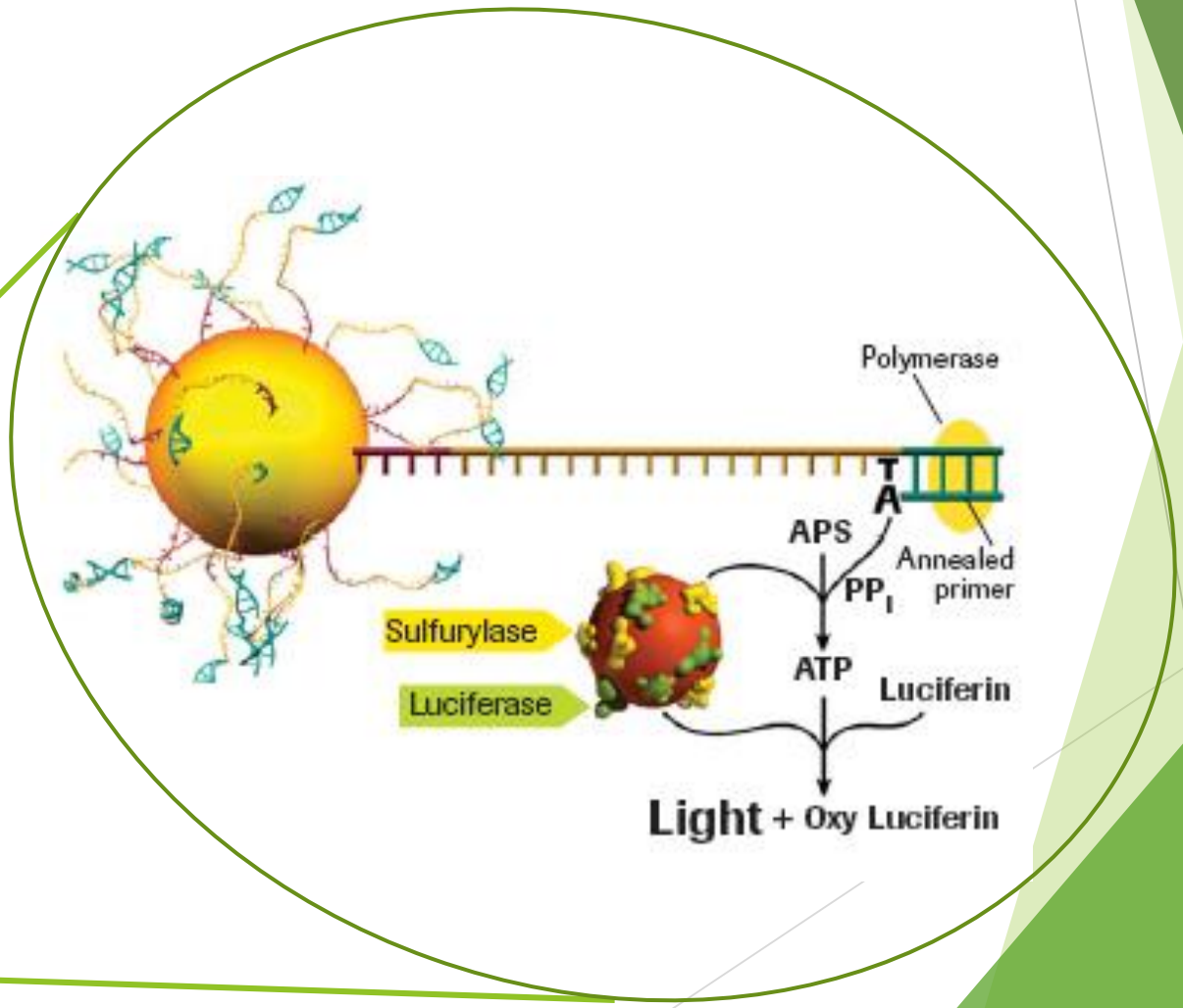


PicoTiterPlate loading

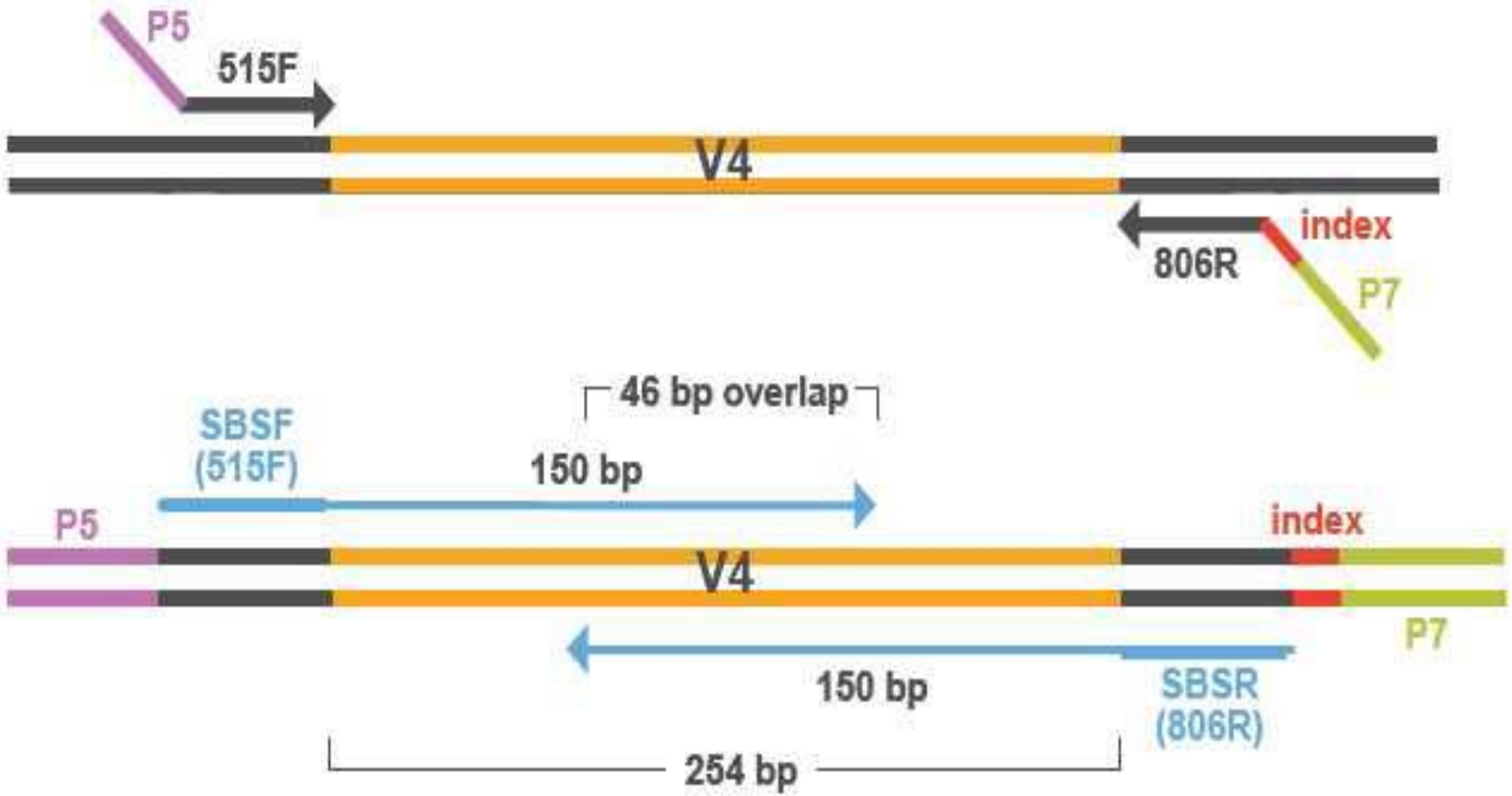




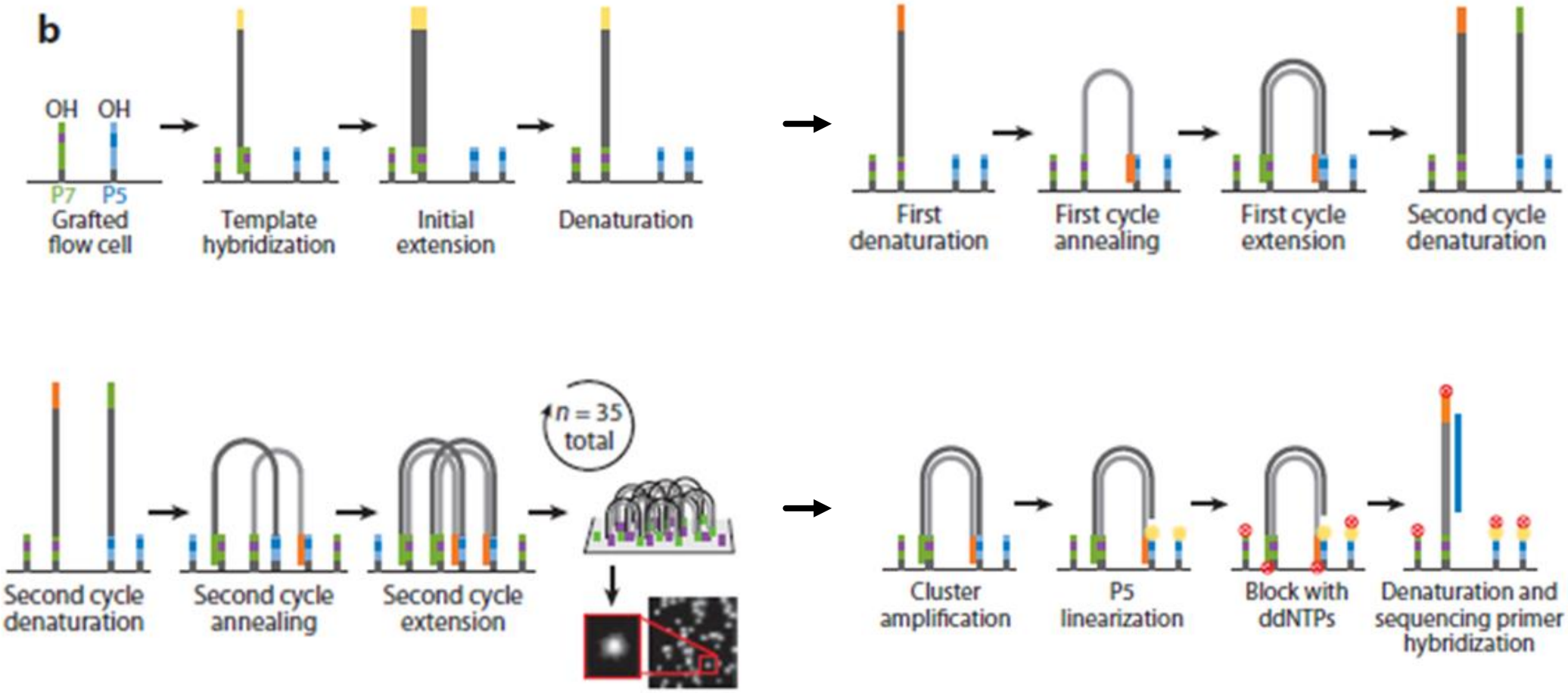
sequencing



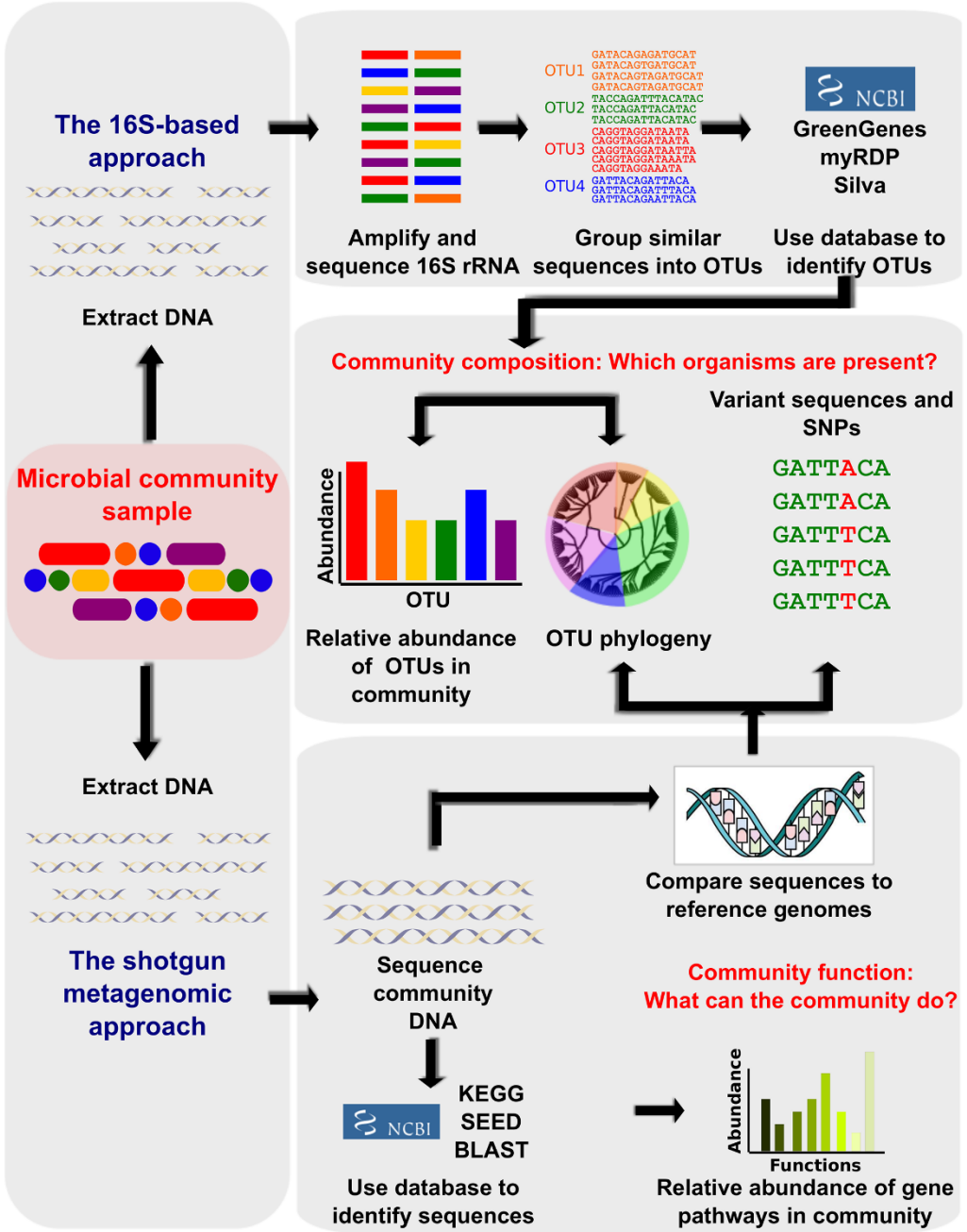
Illumina


P5
515F
V4
index
806R
P7
46 bp overlap
SBSF
(515F)
150 bp
P5
index
V4
P7
150 bp
SBSR
(806R)
254 bp

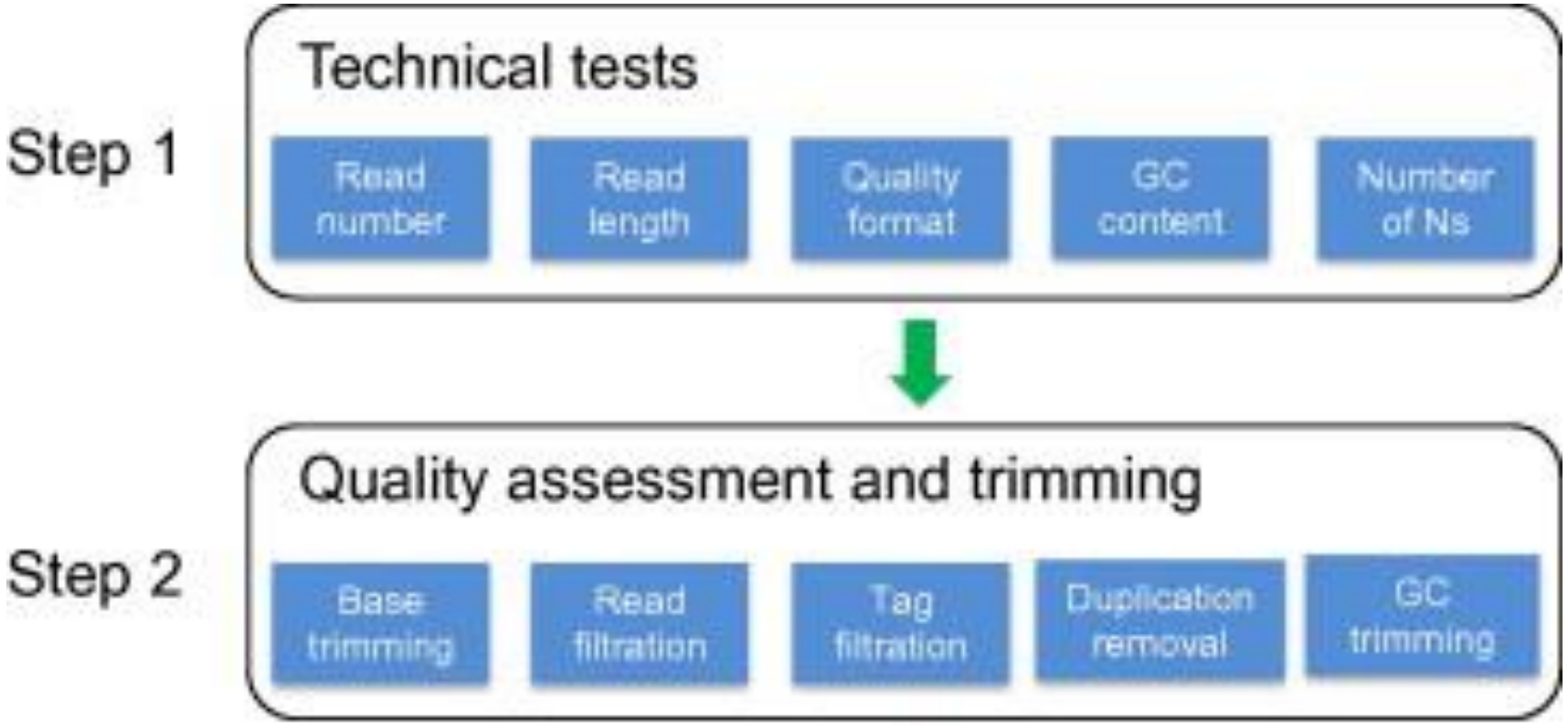
Illumina


b
OH OH
P7 P5
Grafted flow cell
Template hybridization
Initial extension
Denaturation
First denaturation
First cycle annealing
First cycle extension
Second cycle denaturation
Second cycle denaturation
Second cycle annealing
Second cycle extension
n = 35 total
Cluster amplification
P5 linearization
Block with ddNTPs
Denaturation and sequencing primer hybridization

Bioinformatic methods for metagenomics



Evaluation of Reads Quality



Onces we have «cleaned» sequences we have to:

Databases

1) Clusterize in OTUs: Operational Taxonomic Unit is an operational definition to clusterize metagenomic sequences. Cause most part of microorganisms are not cultivable, we compare each reads obtained to each other and all the reads that have a similarity level higher than a preset threshold (generally 97%) are clusterized togheter.

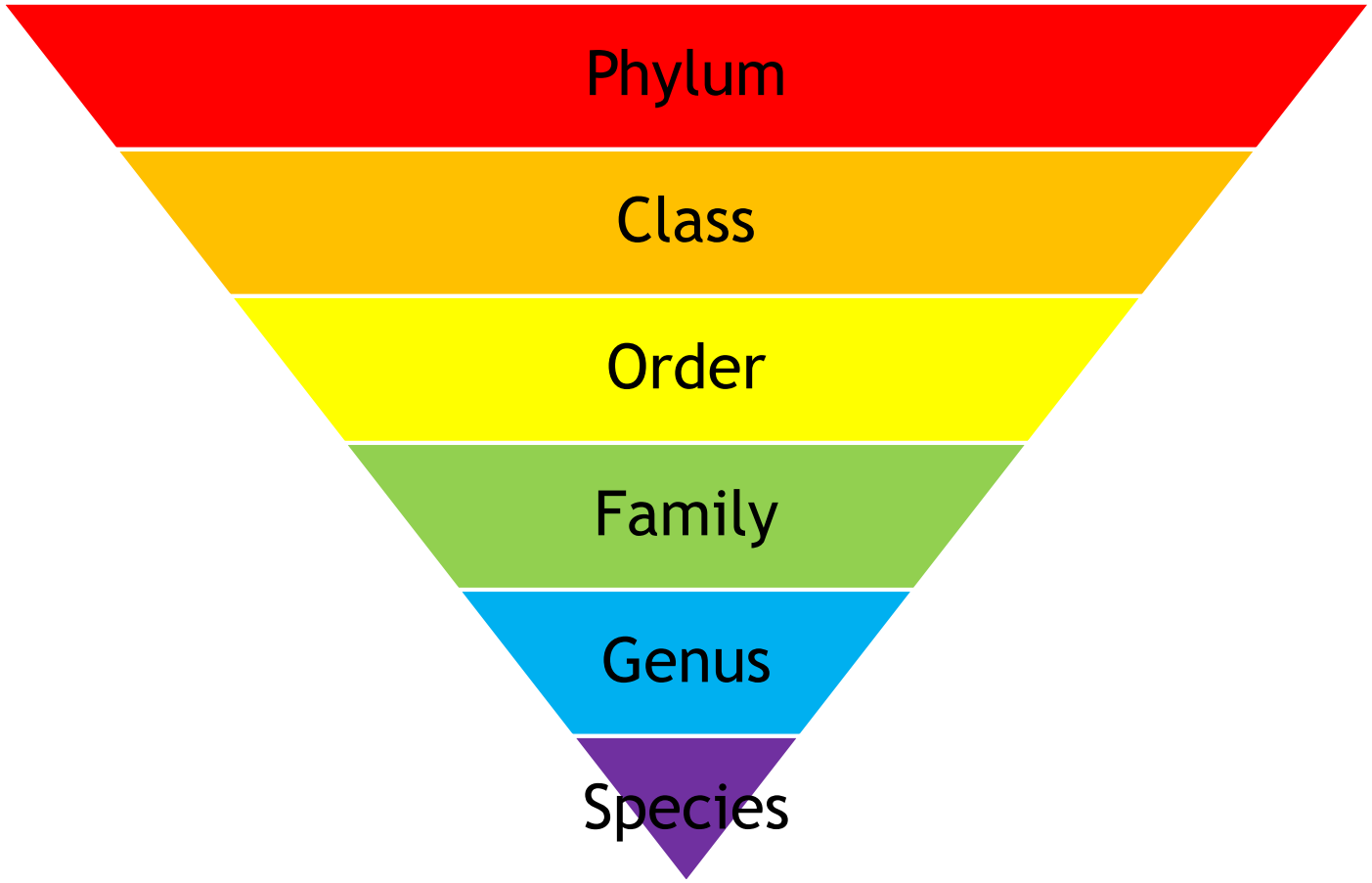
#OTU ID	F3D0	F3D141	F3D142	F3D143	F3D144	F3D145	F3D146	F3D147
OTU_6	749	535	313	372	607	849	493	2025
OTU_25	29	57	14	2	14	22	16	127
OTU_1	613	497	312	247	472	719	349	1720
OTU_8	426	378	255	237	382	627	330	1417
OTU_31	149	38	10	19	25	21	43	31
OTU_2	366	392	327	185	313	542	248	1367
OTU_7	196	370	92	107	48	155	74	105
OTU_10	46	169	87	109	171	209	120	864
OTU_80	26	6	0	1	4	8	18	11

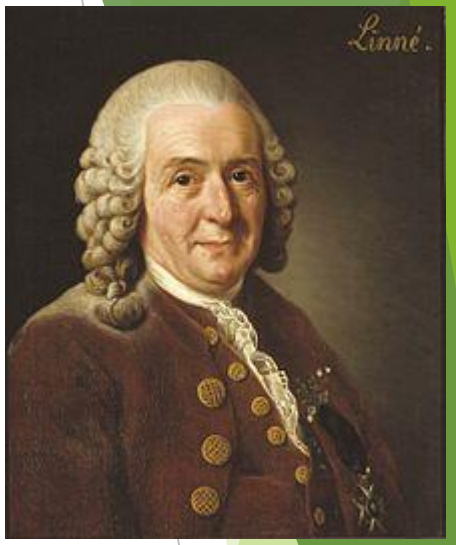
2) After that we chose a representative sequence for each OTUs and that will be alligned to a reference dataset to get the taxonomical classification of that OTU

silva
high quality ribosomal RNA databases

GREENGENES
The 16S rRNA Gene Database and Tools

Taxonomy: Is the science of defining and naming groups of biological organisms on the base of their shared characteristics





Carl Linnaeus (1707 –1778)

Several informatic pipelines have been developed to analyse metagenomics data

Each one with different features → New discoveries in the field are often integrated within these pipelines

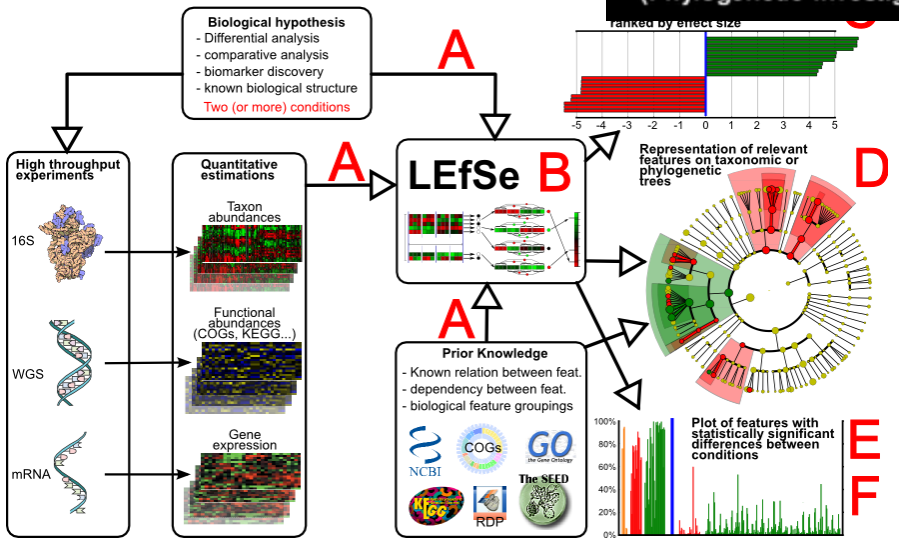
Some of them are useful just in some passage of metagenomic analysis while other contains most of the principles steps of analysis

Quantitative Insights Into Microbial Ecology

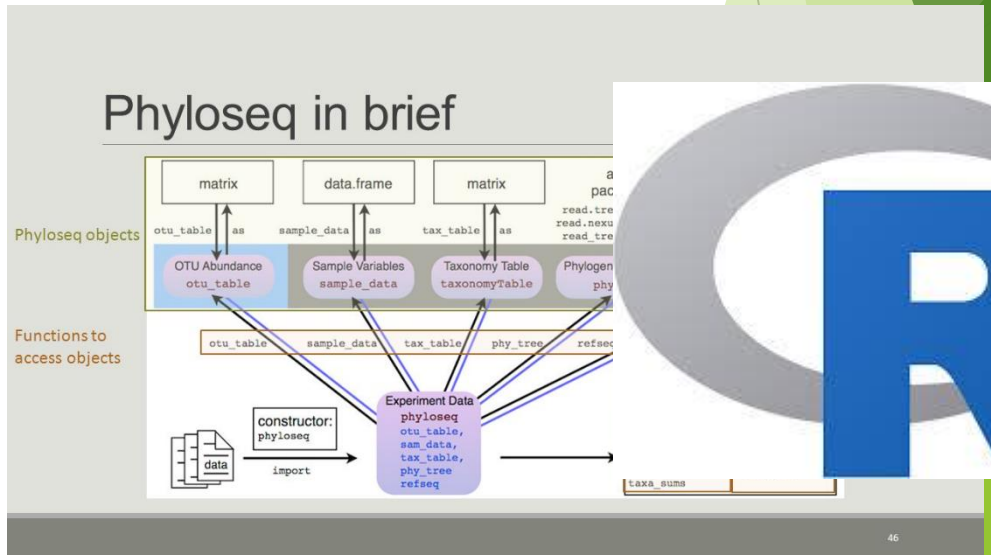
qiime

PICRUSt

(Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)



Mothur



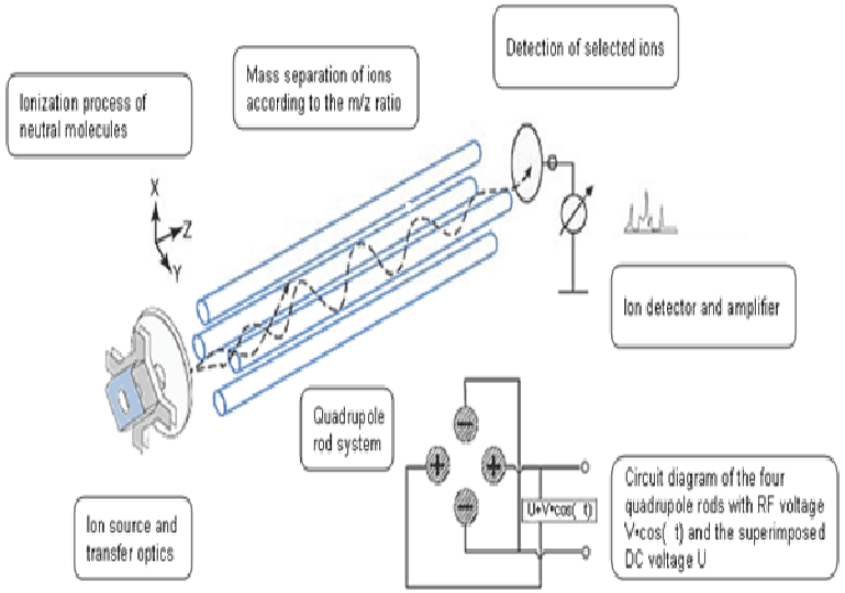
Ecology

- α Diversity
  - Phylogenetic Diversity: It describes the existing biodiviersity within a same area
  - Taxon-based:
    - observed # species (richness)
    - Correct for undersampling (Chao1, Ace)
    - Richness + evenness (Shannon-Weaver index)
- β Diversity: It describes the differences among different area
  - Test if samples have significantly different membership.
    - UniFrac Significance, P test, Libshuff (Phylogenetic)
  - Identify environmental variables associated with differences between many samples.
    - Phylogenetic
      - Unweighted and Weighted UniFrac
      - DPCoA
    - Taxon-based: Jaccard/Sorenson indices

These ecological indices are now used even in metagenomic study to describe the microbial community living within human gut.
Indeed we can consider the human gut as an ecological area where different species of microorganisms are living togheter.

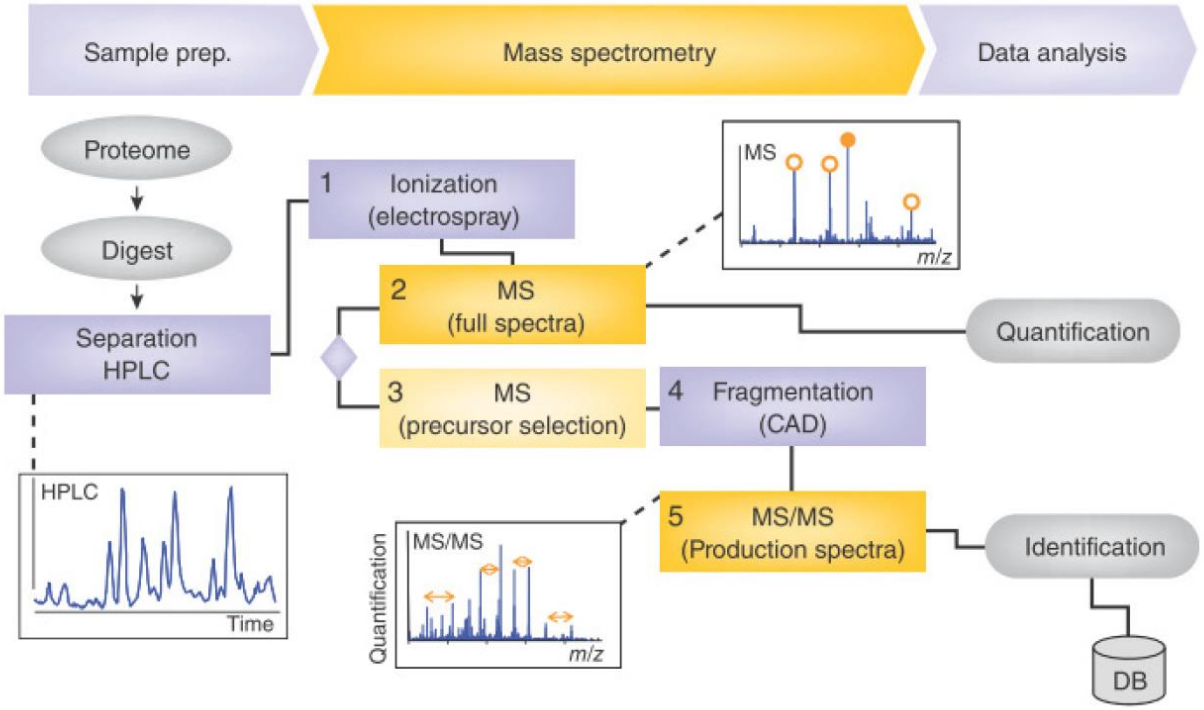
Thus, taken two groups of samples (such as patients and control group) we can use both indices to describe how is the variability within a same group (alpha-diversity) and between the two groups (beta-diversity)

Mass Spectrometry



- Different compounds can be uniquely identified by their mass
  - For small organic molecules the MW can be determined to within 1 ppm or 0.0001% which is sufficiently accurate to confirm the molecular formula from mass alone
  - For large biomolecules the MW can be routinely determined within an accuracy of 0.002% (i.e. within 1 Da for a 40 kD protein)

From Proteomic to Metaproteomic



Domon and Aebersold, Nat Biotechnol 2010

- ✓ How the population is composed
  - Operational Taxonomic Units (OTUs)
- ✓ What function does it accomplish
  - Protein Expression
  - Metabolism
- ✓ How does it react to external factors
  - Drugs
  - Diet
- ✓ How does the host respond to the community changes
  - Wellness
  - Disease

Metabolomics

Metabolomics has been defined as the identification and quantification of all metabolites (chemical fingerprints given by specific cellular processes) in a biological system.

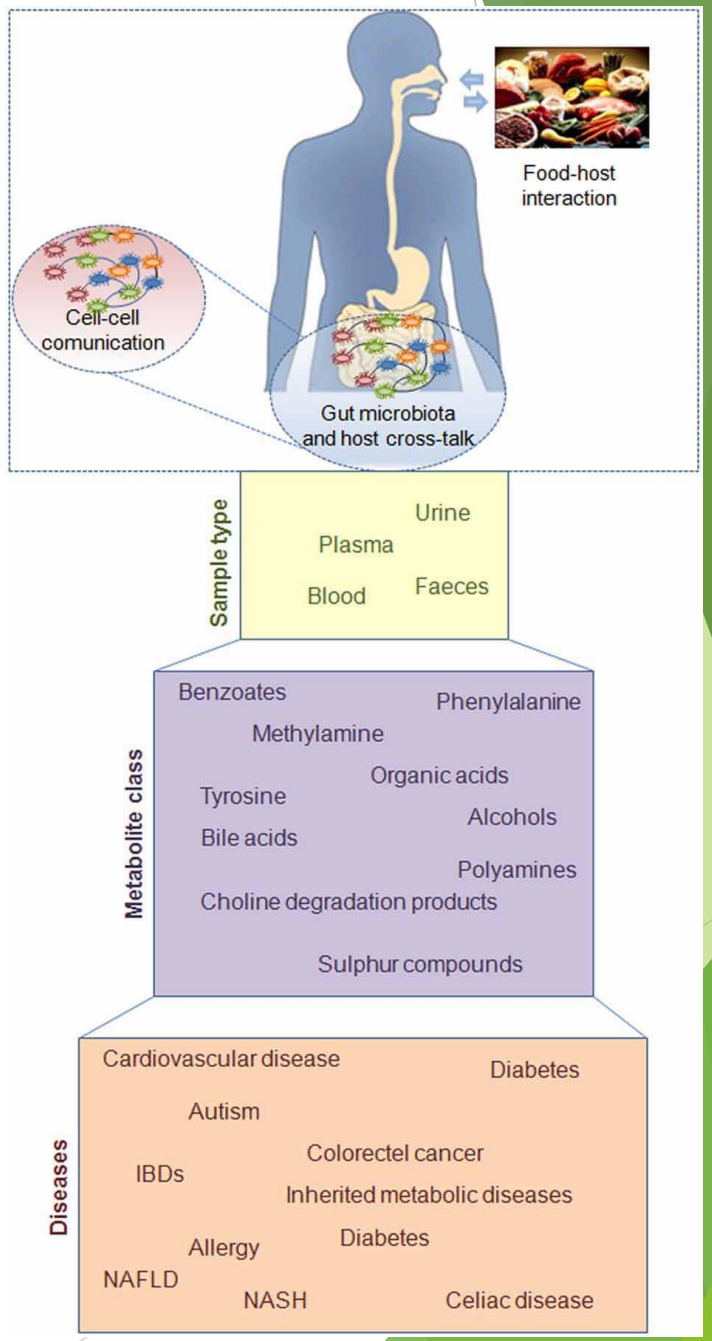
Metabolome refers to the complete set of small-molecules, metabolites (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) to be found within a biological sample, such as a single organism.

Host-microbiome metabolic interaction and cell-cell communication

Diet and caring (*i.e* probiotic consumption, antibiotic treatments, etc) has a key role in the gut microbiota modulation and shaping

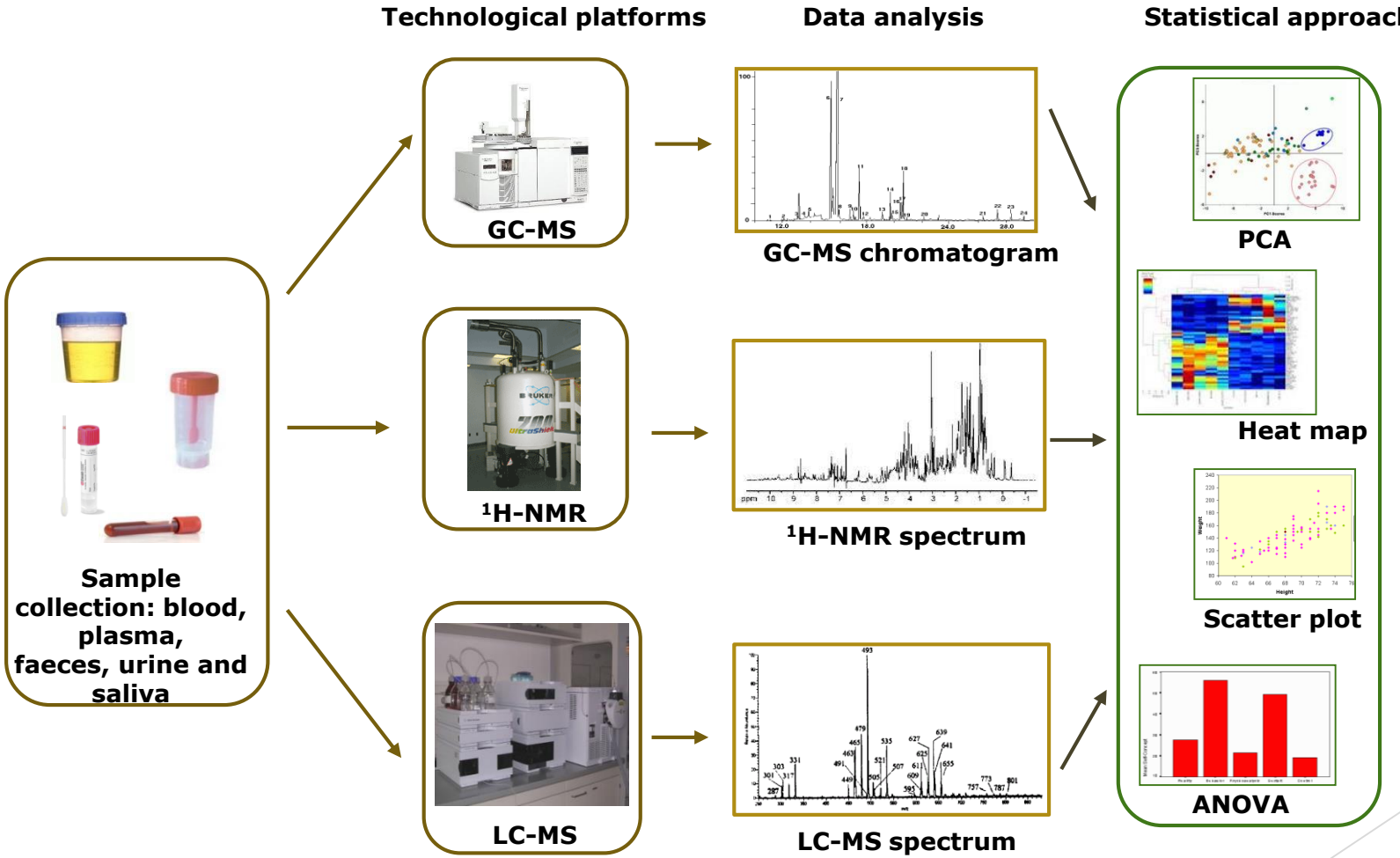
Foods or their ingredients and "drugs" play a crucial role in microbe selection and in a metabolic signaling network construction

The chemical dialogue via low molecular weight metabolites, peptides, and proteins between cell-cell and host-microbes leads to the *metabolite production* which may influence host healthy status ……and possibly represent disease biomarkers.



Vernocchi *et al.*, 2012. Front Cell Infect Microbiol. 2012;2:156

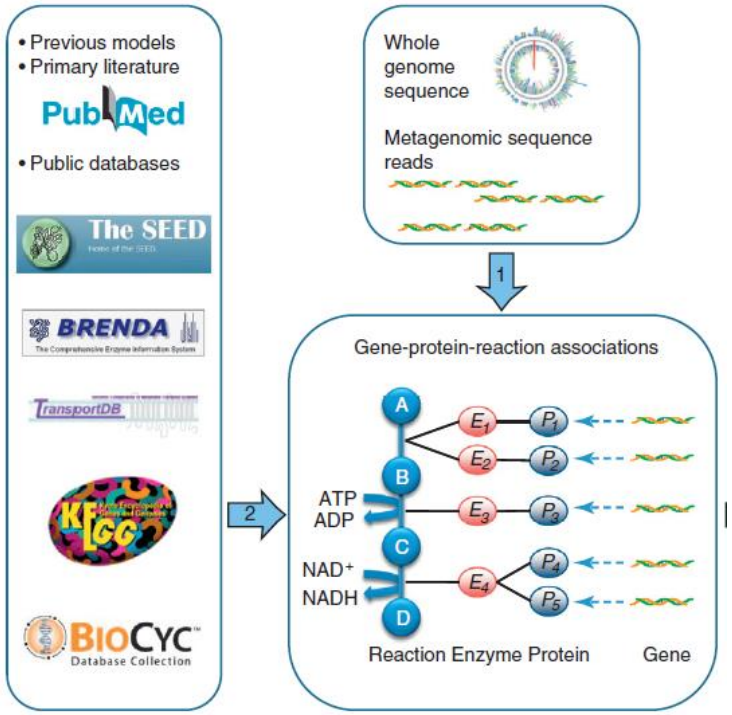
METABOLOMICS



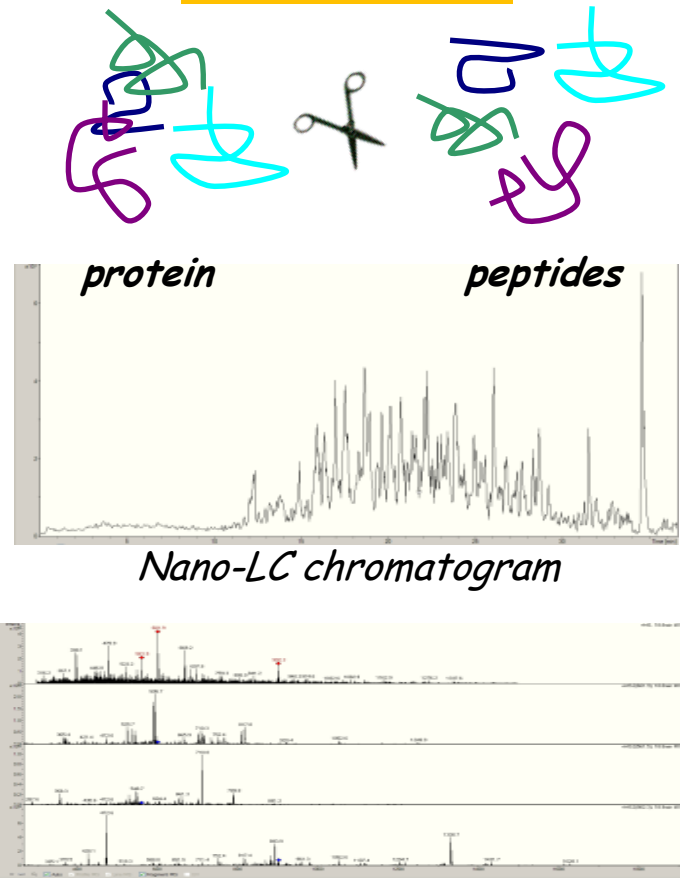
Del Chierico, Gnani, Vernocchi et al., 2014. Meta-omic platforms to assist in the understanding of NAFLD gut microbiota alterations: tools and applications. ***Int J Mol Sci***. 2014 Jan 7;15(1):684-711

INTEGRATED APPROACH: DEVELOPMENT OF ORIGINAL PIPELINES

METAGENOMICS



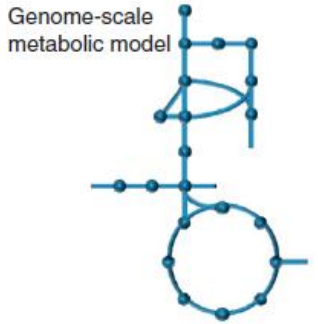
METAPROTEOMICS



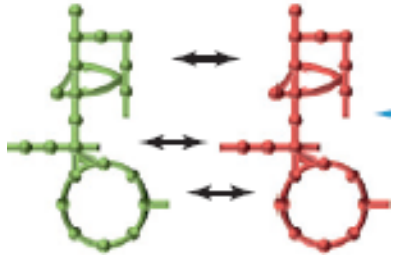
Nano-LC chromatogram

MS and MS² spectra

METABOLOMICS

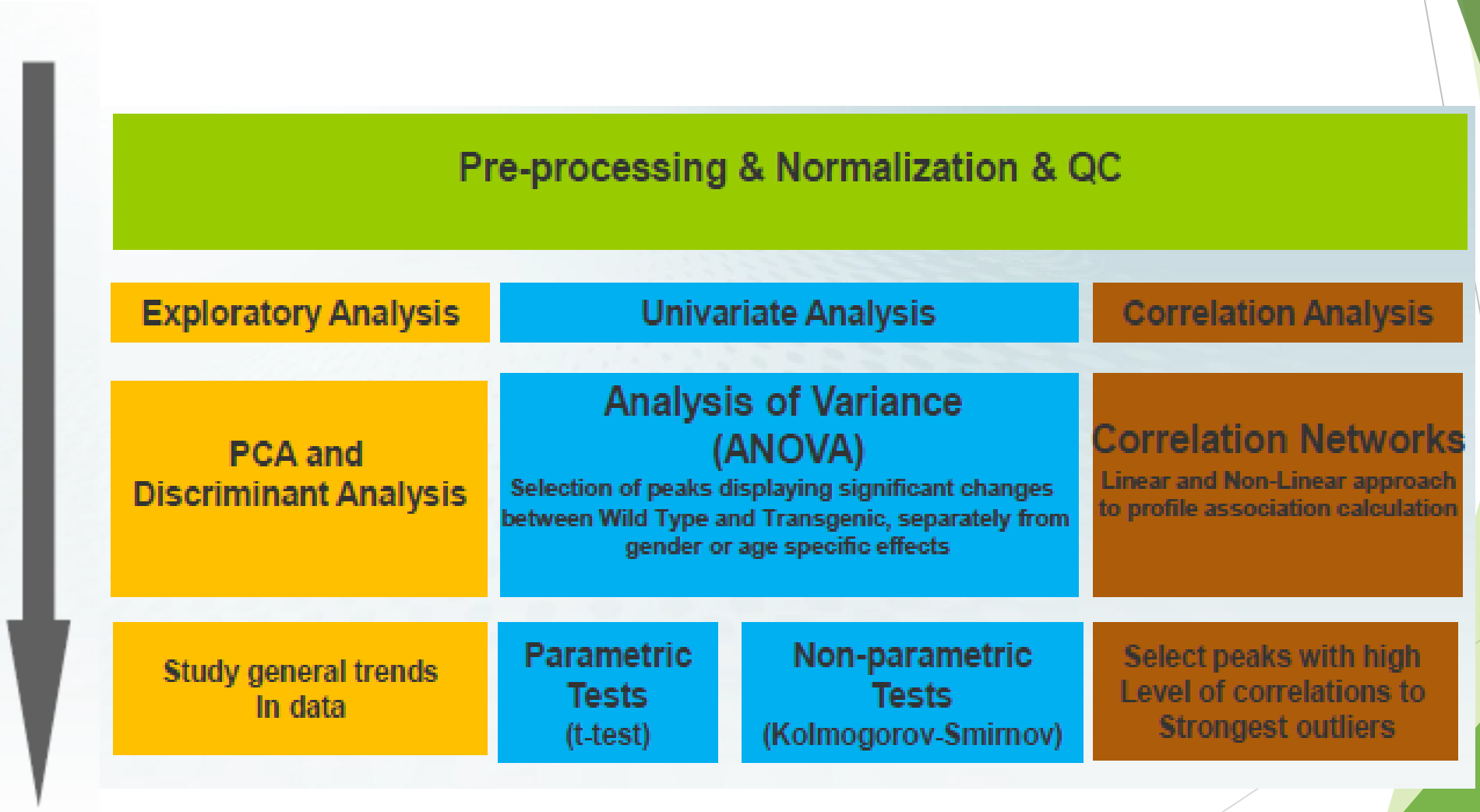


Inter-species metabolite exchange



Del Chierico F, Vernocchi P, et al. Early-life gut microbiota under physiological and pathological conditions: the central role of combined meta-omics-based approaches. ***J Proteomics***. 2012 Aug 3;75(15):4580-7.

DATA ANALYSIS



WHY WE STUDY THE MICROBIOTA BY SYSTEMS BIOLOGY APPROACH?

- Description of microbiota charts in physiological condition
- Description of microbiota charts in pathological condition
- Discovery of microbial biomarker in different diseases
- Discovery of molecular biomarker in different diseases
- Discovery of the interplay between human and microbes
- how do microbial communities work and how are stable

FECAL MICROBIOTA TRANSPLANTATION (FMT)

FMT è un trattamento medico non farmacologico in fase sperimentale

Lo scopo di questa innovativa terapia è quello di ripristinare l'ecologia microbica e l'omeostasi del colon, reintroducendo un microbiota umano «sano» derivante dalle feci di un donatore, in un paziente affetto da patologie associate con l’alterazione della flora batterica intestinale

PROCEDURA

Screening infettivologico del donatore

Produzione della mappa del microbiota del donatore

Raccolta di feci del donatore

Emulsione feci del donatore in soluzione fisiologica

Filtrazione della emulsione per eliminare detriti macroscopici

Introduzione per via rettale in sede endoscopica nel paziente ricevente

Valutazione clinica dell'effetto benefico sul ricevente in follow-up

Valutazione del ripristino del microbiota «sano» nel ricevente mediante mappe del microbiota in follow-up

Valutazione per un successivo FMT