Mikrobiyom Çalışmalarında Kullanılan Laboratuvar Yöntemleri Kullanım Amaçları Kısıtlılıkları

Dr.Füsun Can

KLİMİK İnsan Mikrobiyom ve Biyoterapi Çalışma Grubu Üyesi

KLİMİK
TÜRK KLİNİK MİKROBİYOLOJİ VE İNFEKSİYON HASTALIKLARI DERNEĞİ

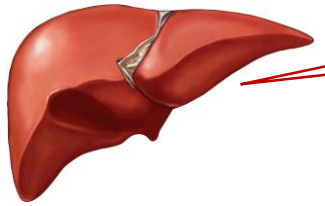
MİBİTÇG
KLİMİK DERNEĞİ MİKROBİYOM VE BİYOLOJİK TEDAVİLER ÇALIŞMA GRUBU

Kısa Bir Hikaye

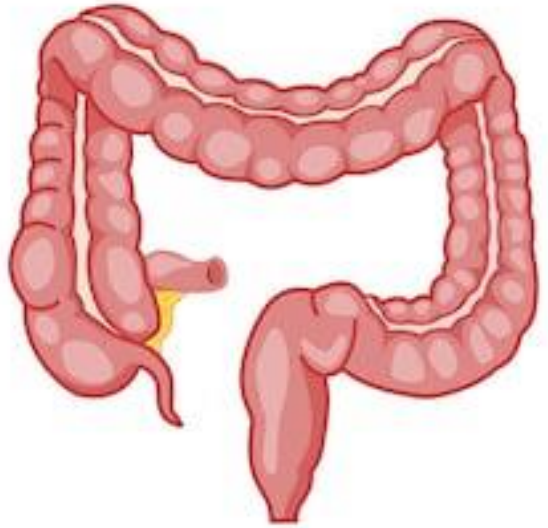
Günlerden bir gün vücut bölümleri hangisinin daha önemli olduğu konıusunda tartışıyorlarmış

Herşey benim kontrolümde

Vücudu çalıştıran benim



Metobolizmayı ben kontrol ederim



Bütün Hastalıklar Barsakta Başlar

Hipokrat MÖ 460-370

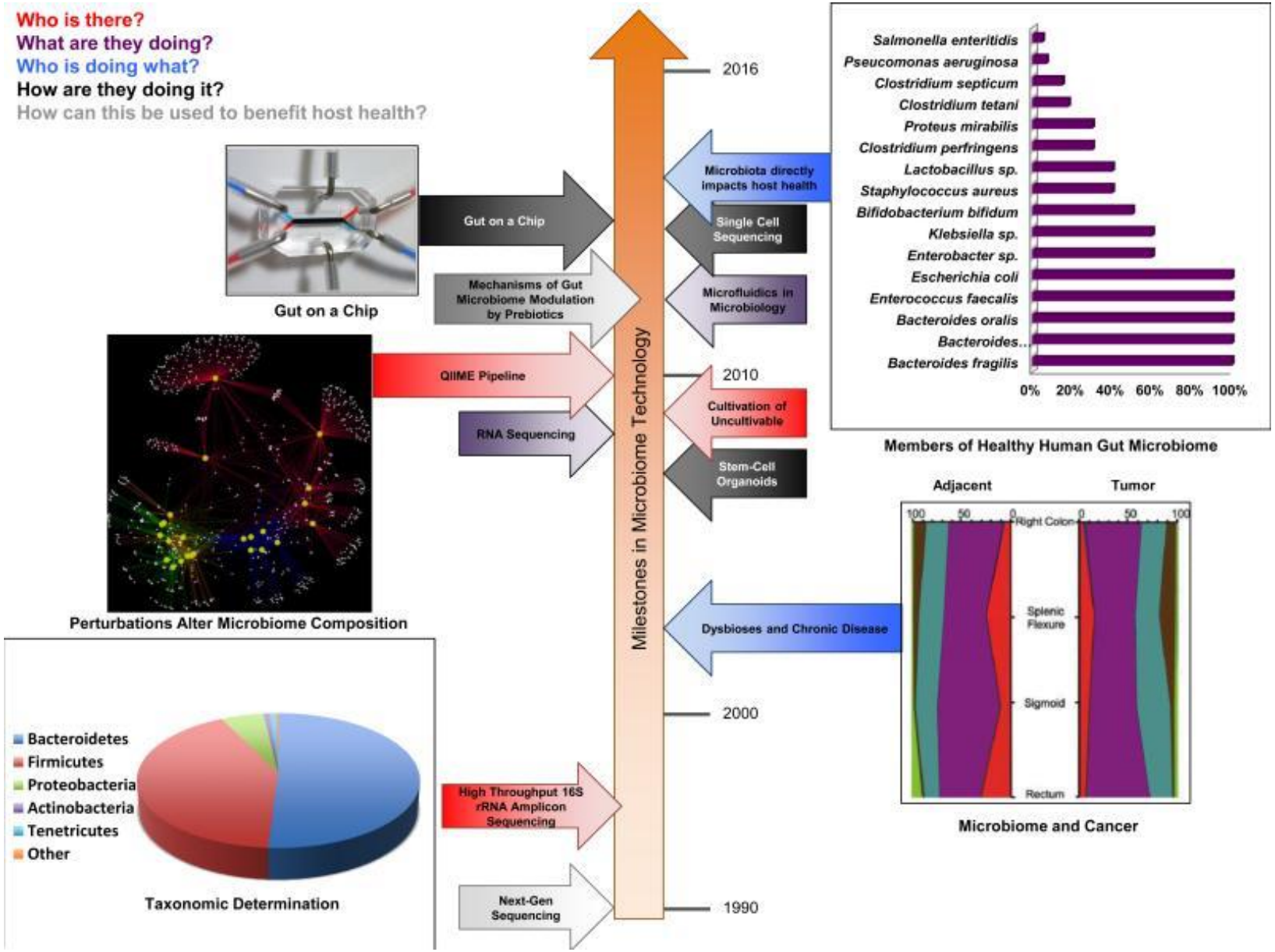
Barsak Mikrobiyatası sağlığın belirleyicisidir

KLİMİK İnsan Mikrobiyom ve Biyoterapi Çalışma Grubu 2019

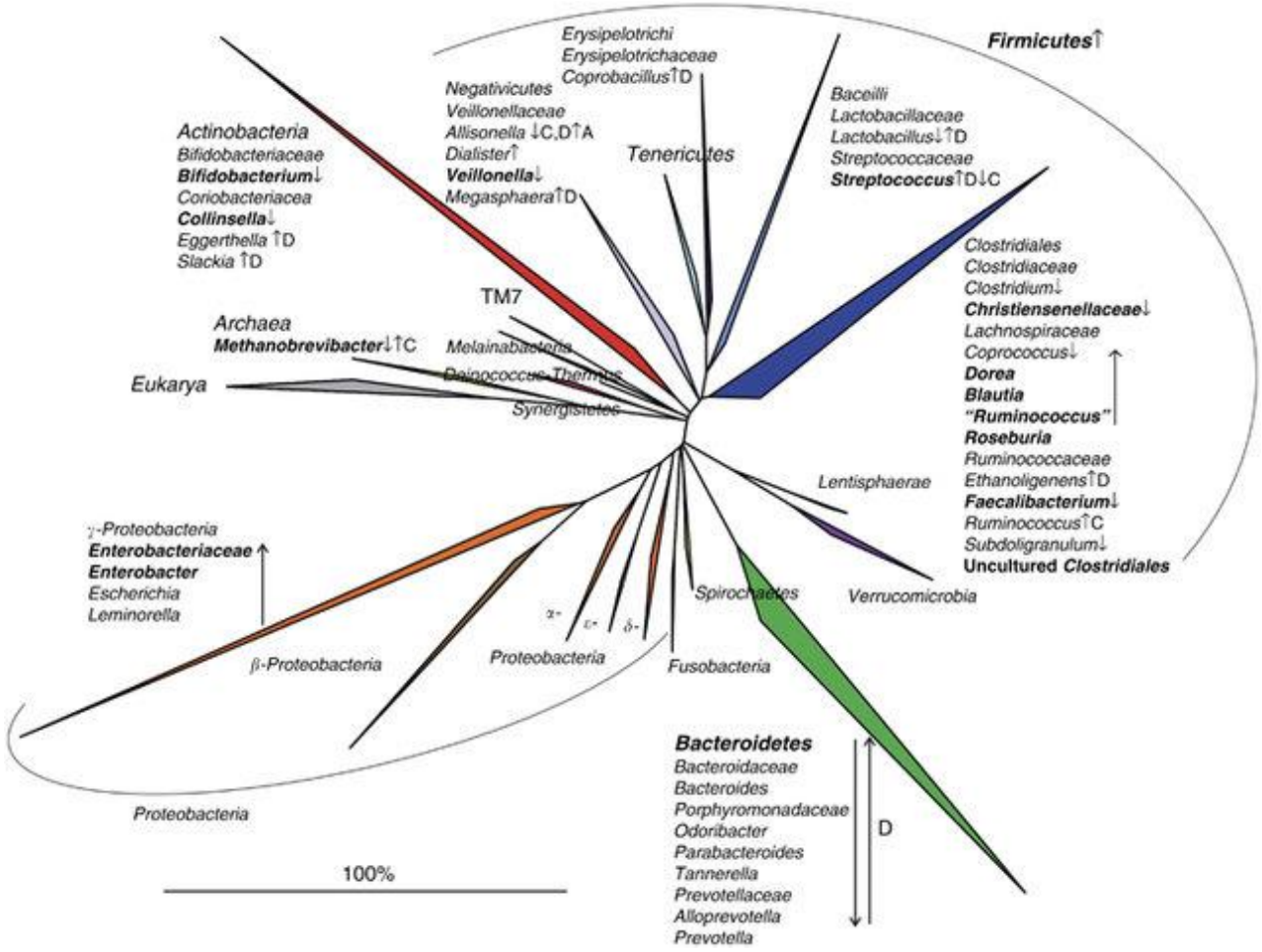
Mikrobiyota çalışmalarının hedefi

- Tiplendirme
- Kantitasyon
- Aktivasyon
- Fonksiyon

- Metagenom
- Metatranskriptom
- Metaproteomik
- Metabolomik


Who is there?
What are they doing?
Who is doing what?
How are they doing it?
How can this be used to benefit host health?
Milestones in Microbiome Technology
2016
2010
2000
1990
Gut on a Chip
Gut on a Chip
Mechanisms of Gut Microbiome Modulation by Prebiotics
QIIME Pipeline
RNA Sequencing
High Throughput 16S rRNA Amplicon Sequencing
Next-Gen Sequencing
Perturbations Alter Microbiome Composition
Bacteroidetes
Firmicutes
Proteobacteria
Actinobacteria
Tenetricutes
Other
Taxonomic Determination
Microbiota directly impacts host health
Single Cell Sequencing
Microfluidics in Microbiology
Cultivation of Uncultivable
Stem-Cell Organoids
Dysbioses and Chronic Disease
Salmonella enteritidis
Pseucomonas aeruginosa
Clostridium septicum
Clostridium tetani
Proteus mirabilis
Clostridium perfringens
Lactobacillus sp.
Staphylococcus aureus
Bifidobacterium bifidum
Klebsiella sp.
Enterobacter sp.
Escherichia coli
Enterococcus faecalis
Bacteroides oralis
Bacteroides...
Bacteroides fragilis
0% 20% 40% 60% 80% 100%
Members of Healthy Human Gut Microbiome
Adjacent
Tumor
Right Colon
Splenic Flexure
Sigmoid
Rectum
Microbiome and Cancer

Barsak Mikrobiyotası Filogenetik sınıflandırması



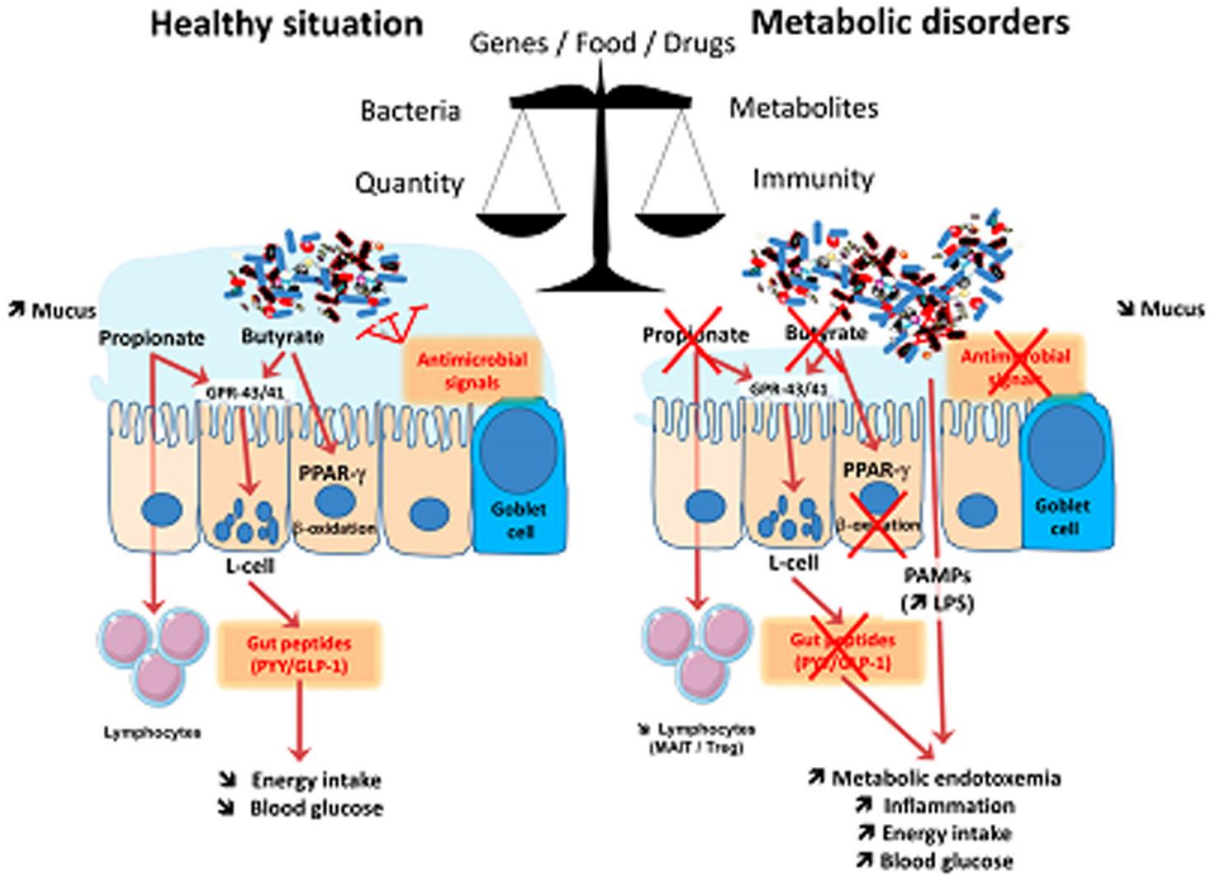
- Bakteriler
- Mantarlar
- Virüsler
- Archea

Barsak mikrobiyotasında predominant bulunan bakteriler

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

Munoz-Garach A, Diaz-Perdigones C, Tinahones FJ.
Microbiota y diabetes mellitus tipo 2. Endocrinol Nutr. 2016;63:560---568.

Mikrobiyota konak İlişkisi



Credit: Cani PD / Gut 2018 (CC BY 4.0)

Problemler……

- Büyük verilerin üretilmesi ve işlenmesi
- Hastalıklarla ilişkilendirilmesi için kanıtlanması
- Sadece popülasyonun tanımlanması yeterli değil aktivitelerinin gösterilmesi
- Standart sağlıklı popülasyon yok
- Teknik avantajlar ve problemler
- Örnek alma, transport belirsiz

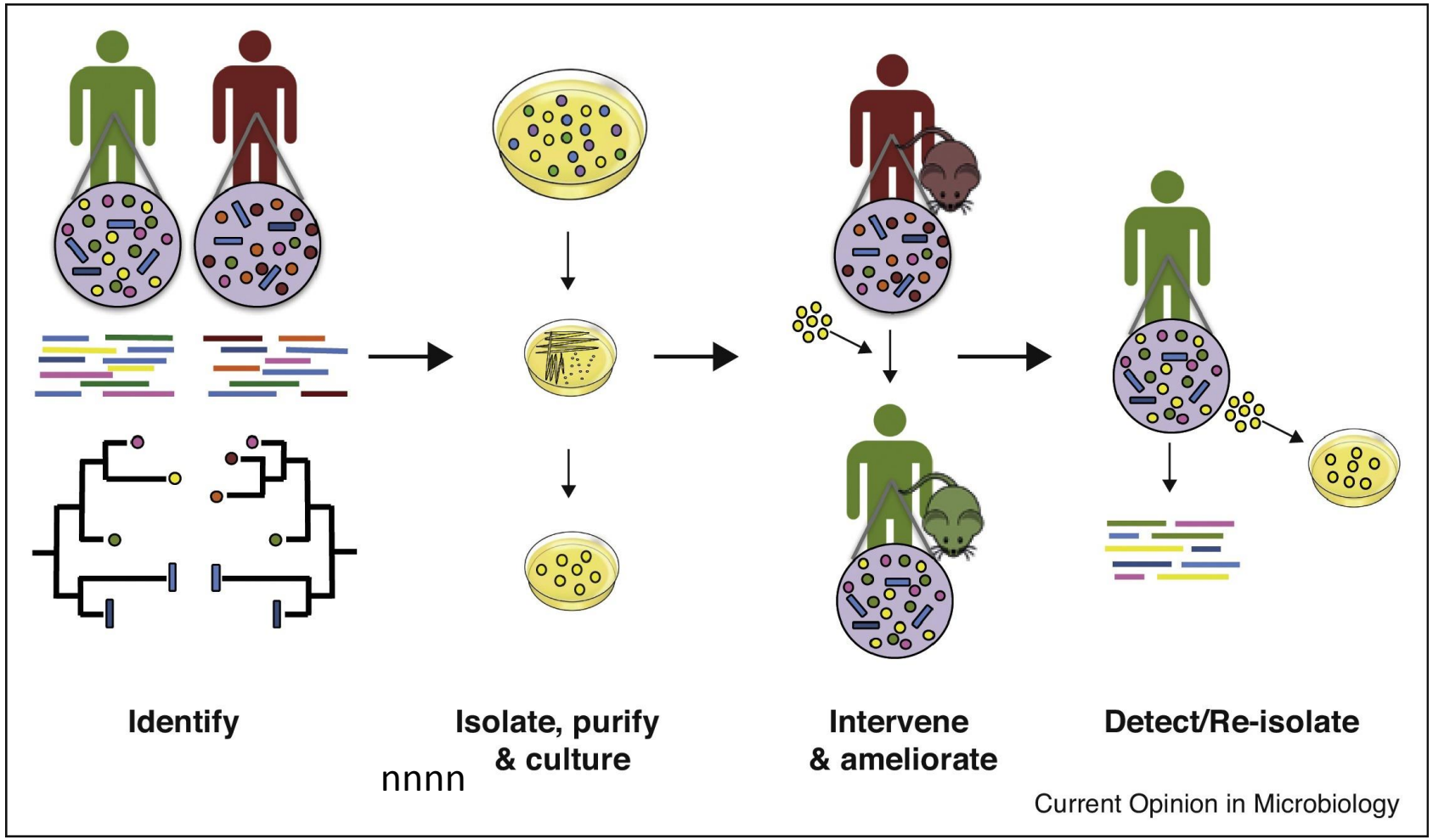

Searching For Ram
ring
ring
ring
ring
ring

1 gr dışkı= 100.000.000.000 bakteri

1 bakteri= 5.ooo.ooo baz (1 milyon byte)

1 gr : 10^{6}x10^{11}= 10 17= 100.000.terabyte

Mikrobiyota çalışmalarında neden sonuç ilişkisi: Koch's postulatı



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Fekal mikrobiyota çalışmaları

- Örnek:
  - Dışkı
  - İntestinal biyopsi
  - İntestinal mukozal lavaj
- Çalışma ve kontrol gruplarından örnek alınmalı
- Uygun şartlarda işlenmeli ve saklanmalı

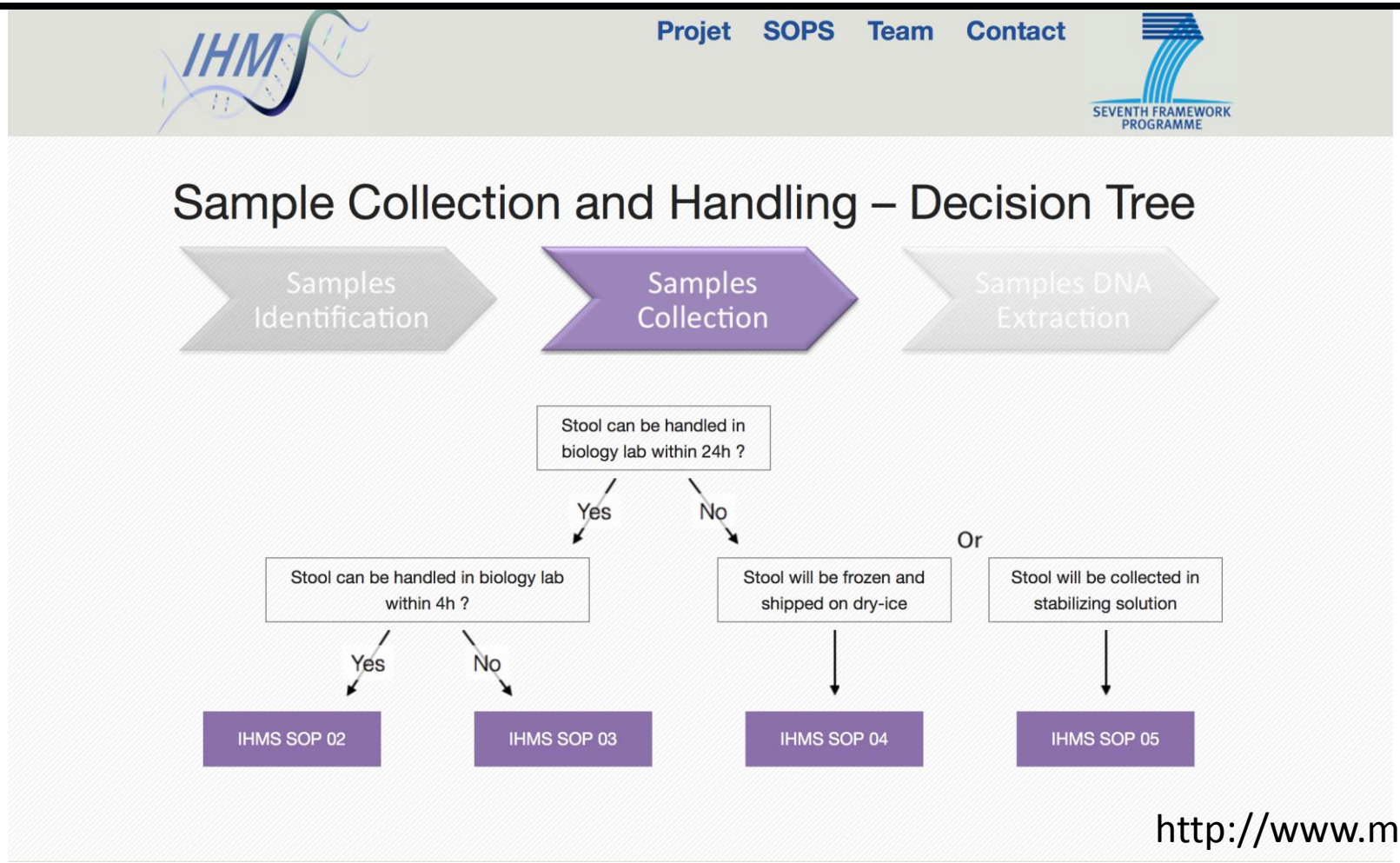
Fekal Örneklerin Hazırlanması

International Human Microbiome Standards Consorsium (IHM)

1- Hand washing is mandatory and essential
2- The fecal sample minimal volume must be at least the volume of a clementine
3- The maximal volume is determined by the volume of the plastic bag (i.) and white pot (ii.)
4- The plastic bag (i.) must NEVER be closed
5- Close the pot (ii.) tightly with the plastic lid (iii.)
6- Stool samples should be kept at room temperature
7- Stool samples should be brought to the 'laboratory' within 4 h after collection for aliquoting and storage at -80°C

(i.)*refers to the picture below cf. 8. Step by step procedure

International Human Microbiome Standards Consorsium (IHM)



http://www.microbiome-standards.org


ii.
iii.
i.

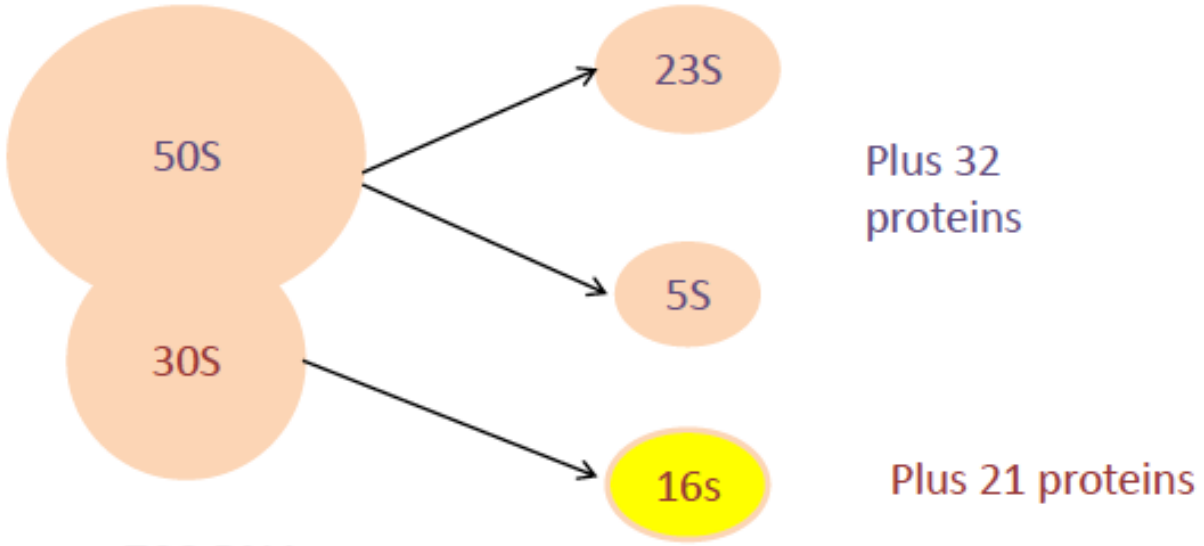

2.

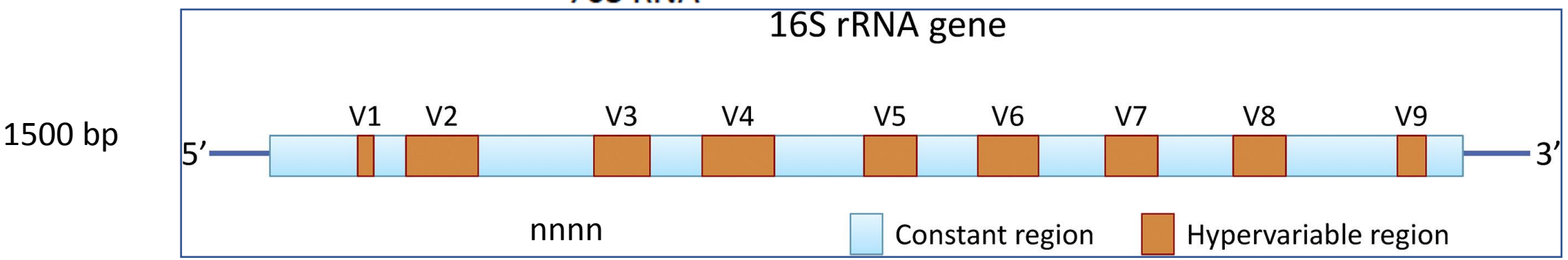

4.


5.

Mikrobiyotanın çalışmalarında kullanılan yöntemler
A. Kültüre Dayalı Yöntemler
B.Moleküler Yöntemler
a. Sekans dışı yöntemler
I. Floresan insitu hibridizasyon flow sitometri
II. Pulse field jel elektroforezi
III: Denatürasyon gradient jel elektroforezi
IV. Isı gradient jel elekroforezi
b. Sekansa dayalı yöntemler I. 16S rRNA ve hipervariable bölgelerin sekansı (Hedefe odaklı sekans)
II. Tüm genom sekansı (metagenom)
III.Tüm RNA sekansı (meta_RNA transkriptom)
C. Küçük metabolitlerin saptanmasına dayalı yöntemler
I. Gaz koromotografisi, Kitle spektrometrisi
II: Kitle spektrometrisine eşlenmiş kapiller elektroforez
III. Nükleer ve proton manyetik rezonans spektrometrisi
IV. Infrared spektrometri

16s rRNA geni mikrobiyota çalışmalarının hedeflerinden biridir





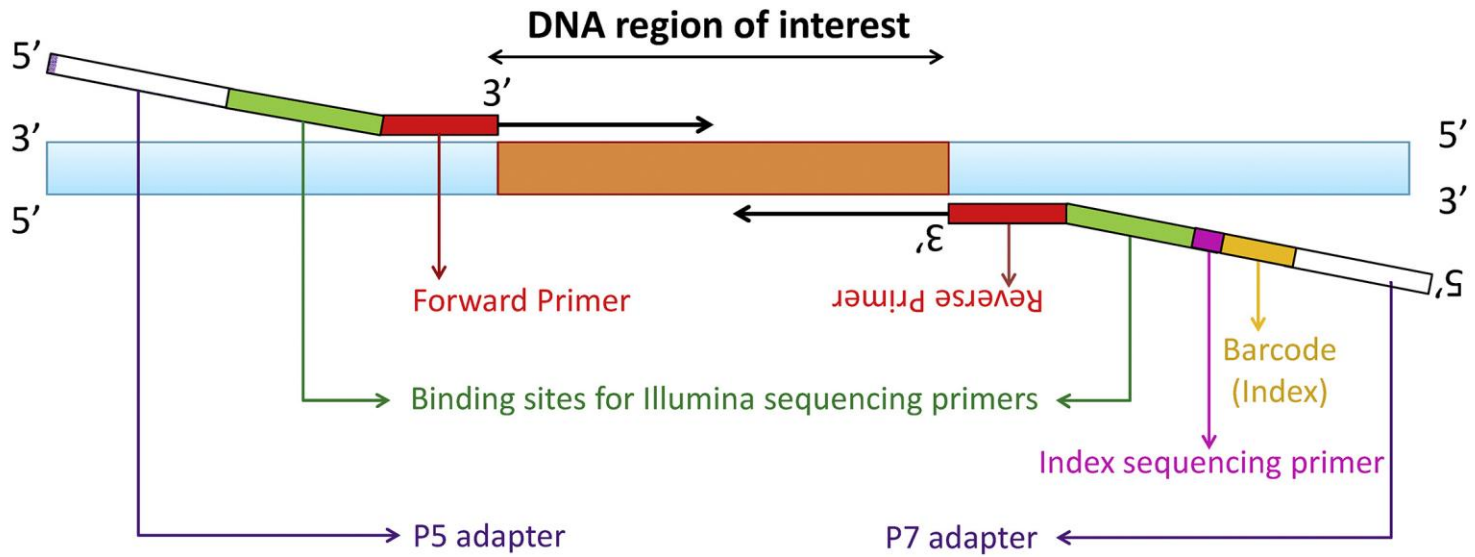
Hipervariable bölgelerin ve kütüphanelerinin seçimi

- 16S rRNA V3, V4 ve V6 bölgeleri
- V3-V4 veya V4-V5 bölgeleri en iyi taksonomik sonuç

- Mümkün olduğunca çok tür
- Ayırım gücü yüksek
- Üretilen uzunluktaki sekansları düşük maliyette okuma

Kütüphanelerin hazırlanması ve sekanslama

A

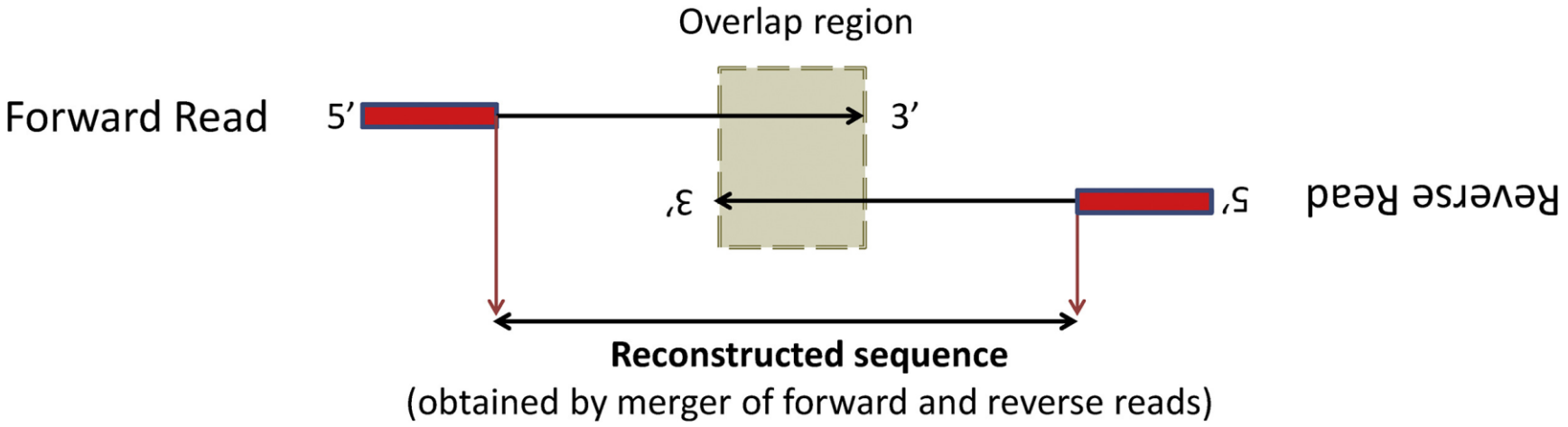


B

Primer name	Primer nucleotide sequence
V3F	5’-aatgatacggcgaccaccgagatct acactctttccctacacgacgctcttccgatct NNNNCCTACGGGAGGCAGCAG-3’
V3R	5’-caagcagaagacggcatacgagat XXXXX gtgactggagttcagacgtgtgctcttccgatct ATTACCGCGGCTGCTGG-3’

Eşleştirme

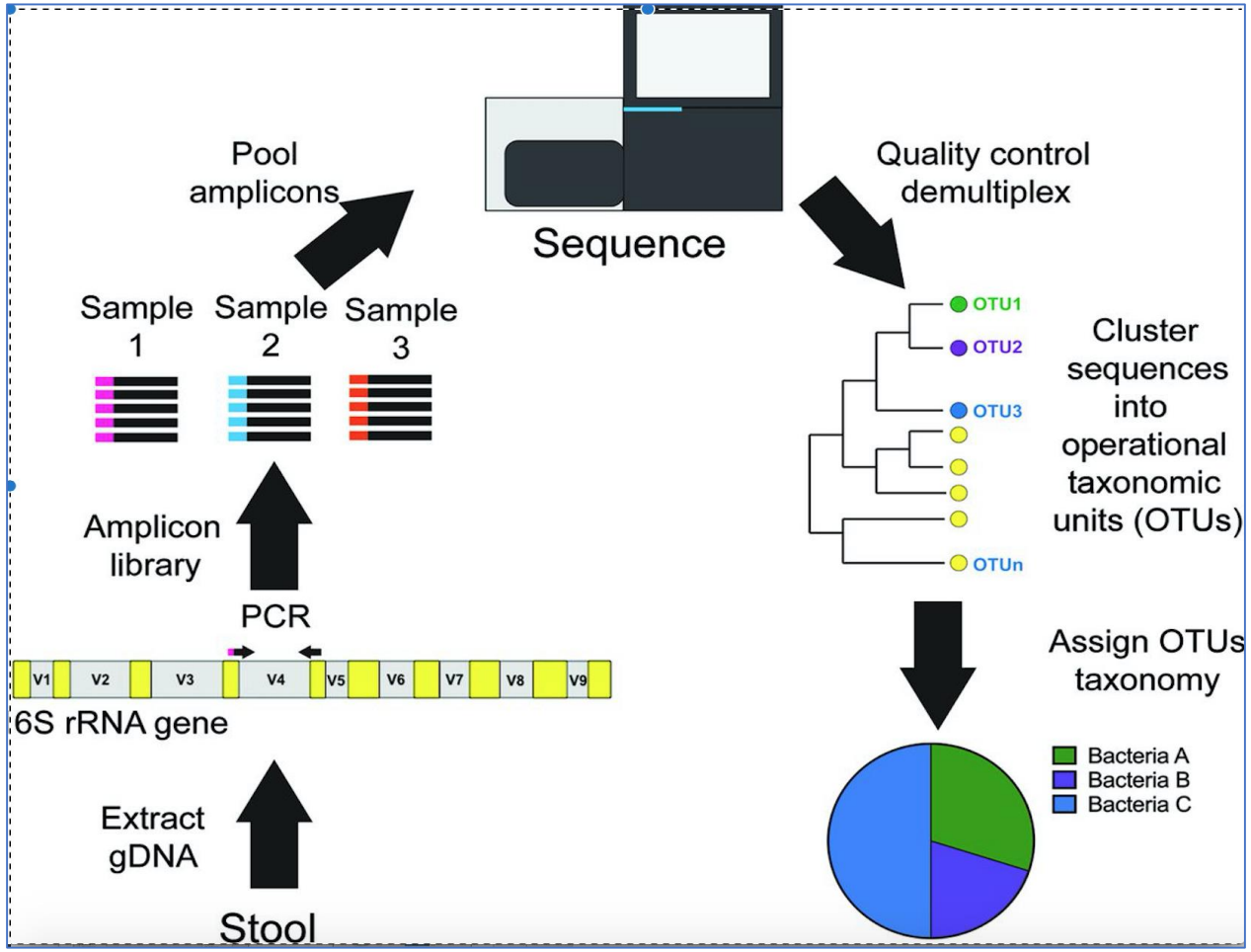
- Adaaptor ve primer sekansları temizlenir
- R ve F okumalar birleştirilir
- 250-550 bp fragmentler



Avantajları:

- Daha uzun nukleotod
- Düşük kaliteli okumalar elenir

16S rRNA Sekanslama



- Masif paralel sekans
  - Küçük fragmentlar (150-300 bp)
- Büyük veri
  - Analiz kabusu
- Gelişen software teknolojisi
  - configs

Kulanılan Teknolojiler

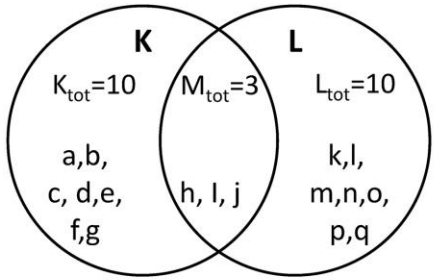
- İlumina
  - Miseq (250-300 baz uzunluğu)
  - Hiseq (150 baz uzunluğu) yüksek kapasiteli
  - Novaseq
  - Çogunlukla başarılı
- Single molecule real time (SMRT) -16S rRNA
- Yaygın değil -Pahalı

Table 2 Popular Bioinformatics Tools Used for 16S rRNA Metagenome Analysis.

Purpose	Tools	URL
Trimming of primers and adapters	Cutadapt	https://github.com/marcelm/cutadapt
	Sickle	https://github.com/najoshi/sickle
	cutPrimers	https://github.com/aakechin/cutPrimers
	AdaperRemoval	https://github.com/MikkelSchubert/adapterremoval
Quality control	NGS-QC ToolKit	http://www.nipgr.res.in/ngsqctoolkit.html
	Trimmomatic	http://www.usadellab.org/cms/?page=trimmomatic
	clinQC	https://sourceforge.net/projects/clinqc/
	AfterQC	https://github.com/OpenGene/AfterQC
Merger of paired-end reads	Pandaseq	https://github.com/neufeld/pandaseq
	PEAR	https://sco.h-its.org/exelixis/web/software/pear/
	FLASH	https://ccb.jhu.edu/software/FLASH/
	MeFiT	https://github.com/nisheth/MeFiT
16S-rRNA metagenome analysis pipelines	QIIME	http://qiime.org/
	MOTHUR	https://www.mothur.org/
	MG-RAST	http://metagenomics.anl.gov/
	MICCA	http://micca.org/

Alfa ve Beta Diversity

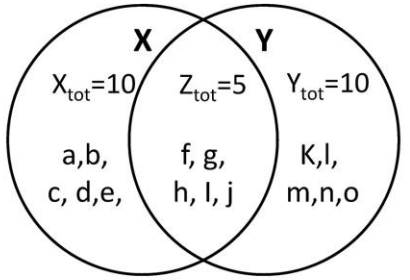
A



Beta Diversity Index : BDI(K,L)

BDI(K,L) = 1-[(M_{tot} X 2) / (K_{tot} + L_{tot})]
= 1-((3*2)/(10+10)
= **0.7**

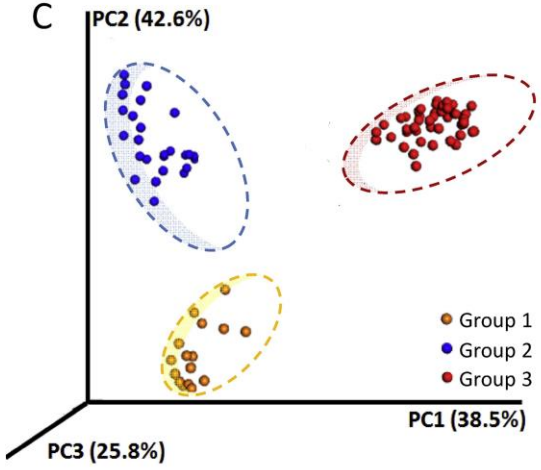
B



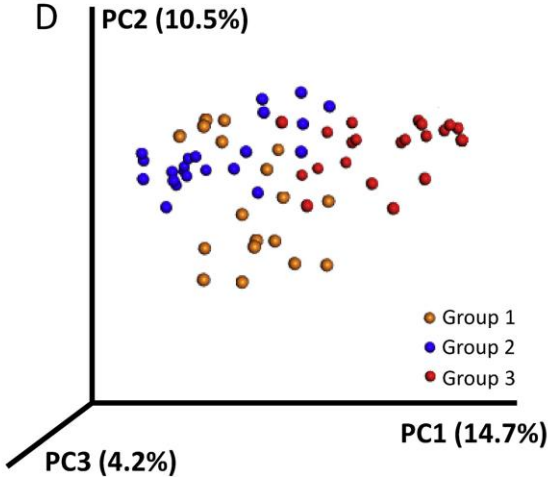
Beta Diversity Index : BDI(X,Y)

BDI(X,Y) = 1-[(Z_{tot} X 2) / (X_{tot} + Y_{tot})]
= 1-((5*2)/(10+10)
= **0.5**

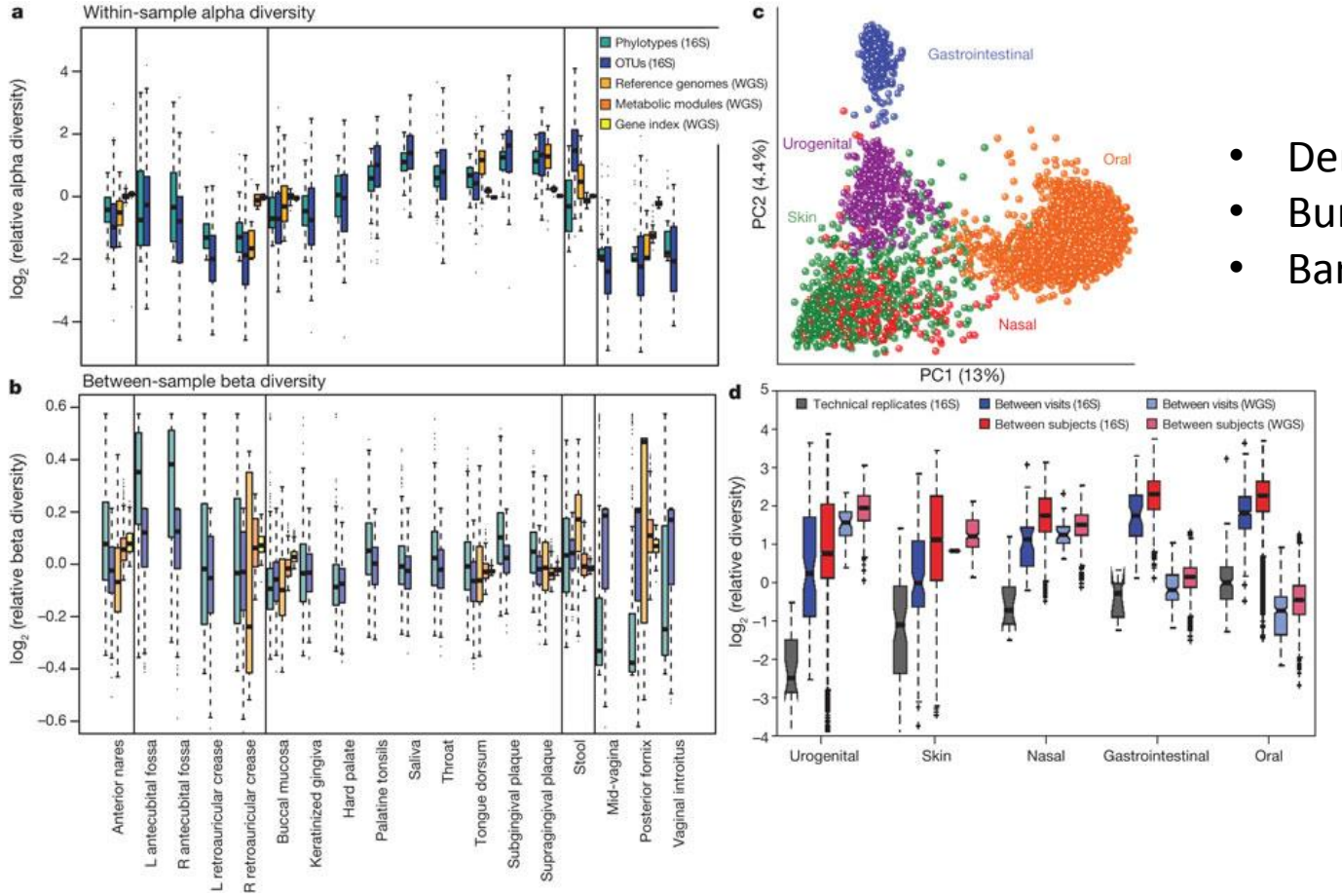
C



D



Diversity of the human microbiome is concordant among measures, unique to each individual, and strongly determined by microbial habitat.



- Deri çok değişken, vagina stabil
- Burun deri ve agız arası geçiş
- Barsak ayrı

C Huttenhower *et al. Nature* **486**, 207-214 (2012) doi:10.1038/nature11234

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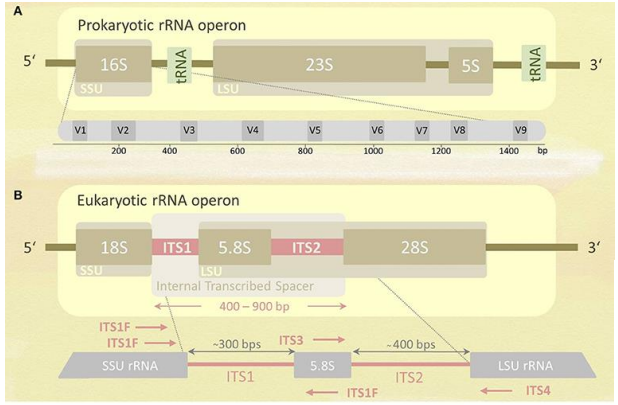
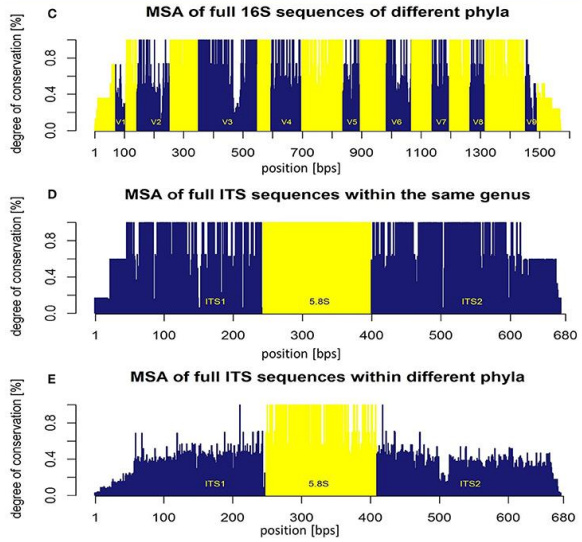
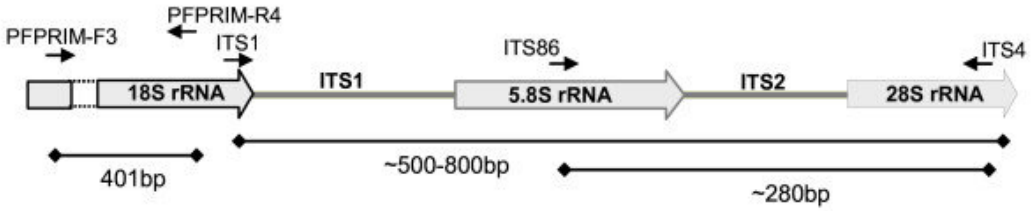
Mycobiota







Veri Analizi

pre-processing
standard procedure
(dependent on sequencing technology)

OTU picking
closed reference
similarity based binning

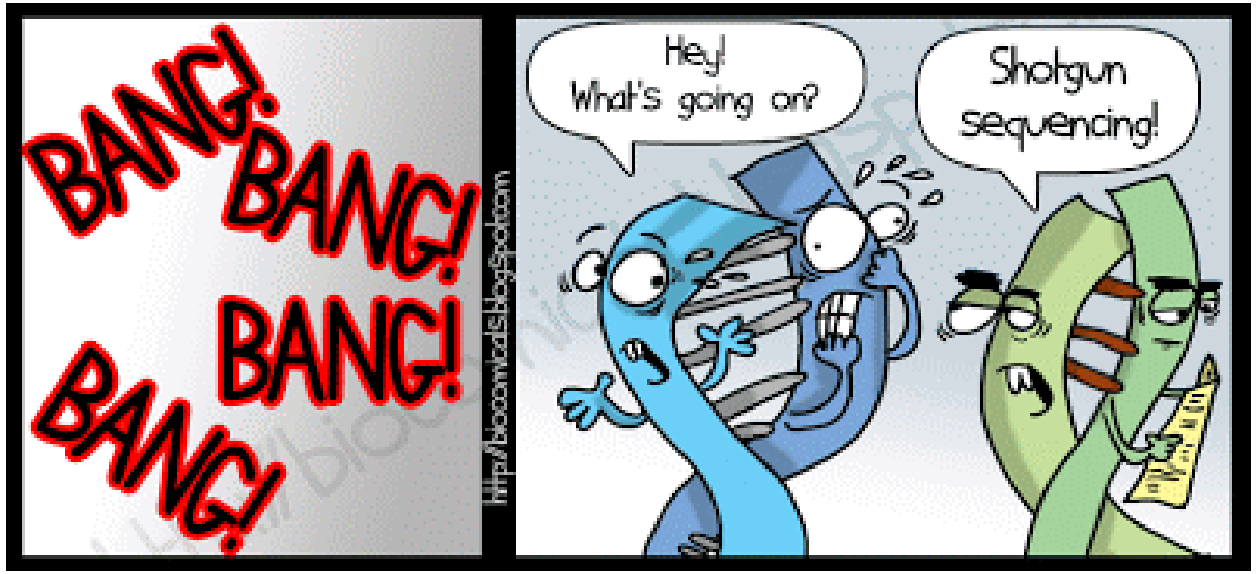
classification
UNITE ITS reference collection
ITS RDP classifier

visualization & statistics
standard techniques
no distance matrix based phylogenetic treeing methods

ShotGun Metagenomik Sekans (MSS)

16S rRNA dsekans dezavantajları

- Sınırlı Sınıflandırma Yapabilme
- Sadece bakteri tanımlayabiliyor
- Fonksiyonel analiz etkinliği sınırlı

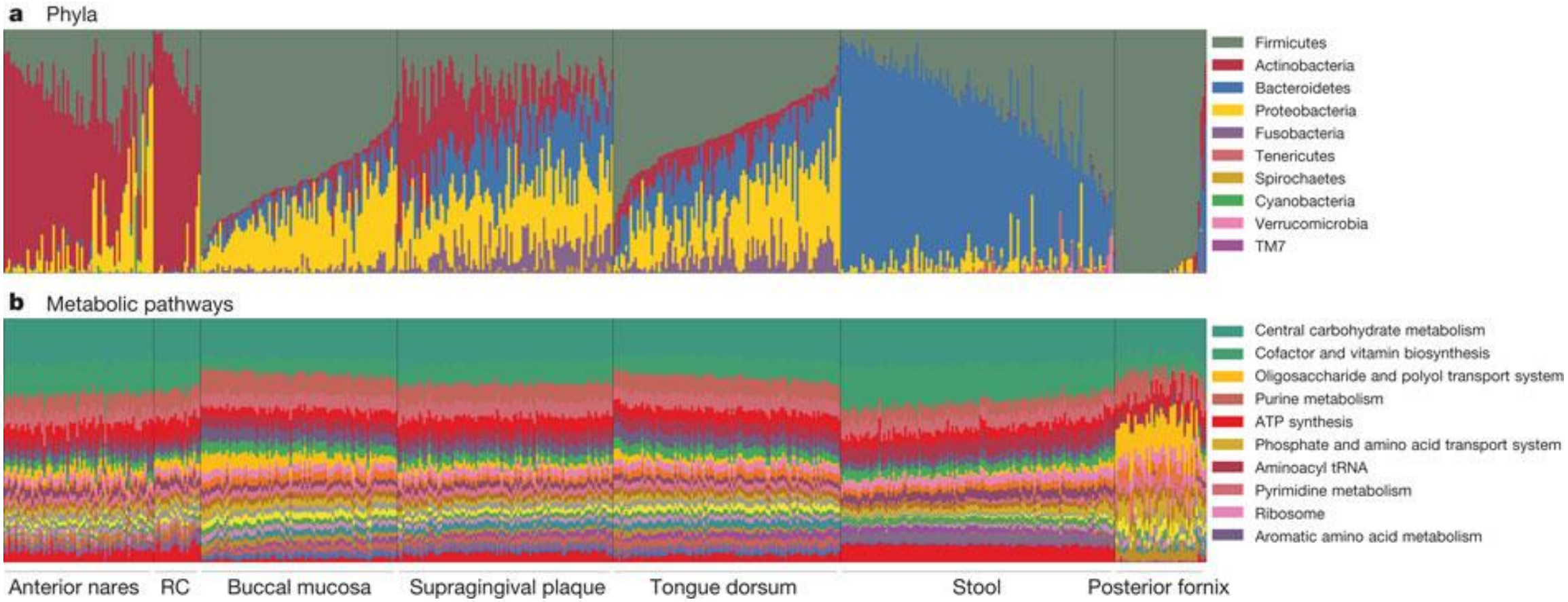


MSS

- Örnekteki tüm genomları görebilme
- Fonksiyonel analiz yapabilme


escherichia
CONJUGATION
does pili size matter?
16 NEW Antibiotic Resistances
for this summer!
special
does your host really love you?
SEXY FLAGELLA
in only 10 days!
TOP10
Animal Guts
to colonize!
PEDROMICS

Carriage of microbial taxa varies while metabolic pathways remain stable within a healthy population.



C Huttenhower *et al. Nature* **486**, 207-214 (2012) doi:10.1038/nature11234

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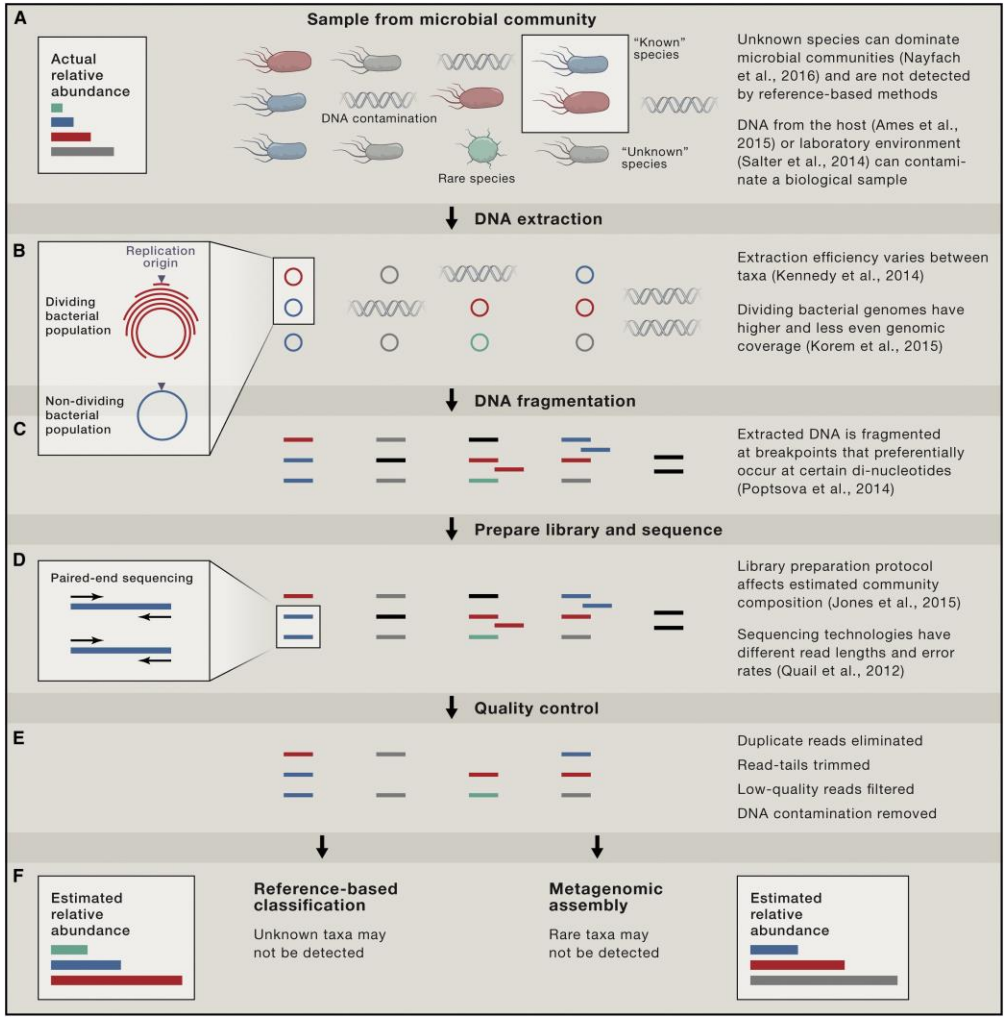
Tam Genom Sekans Çalışmaları

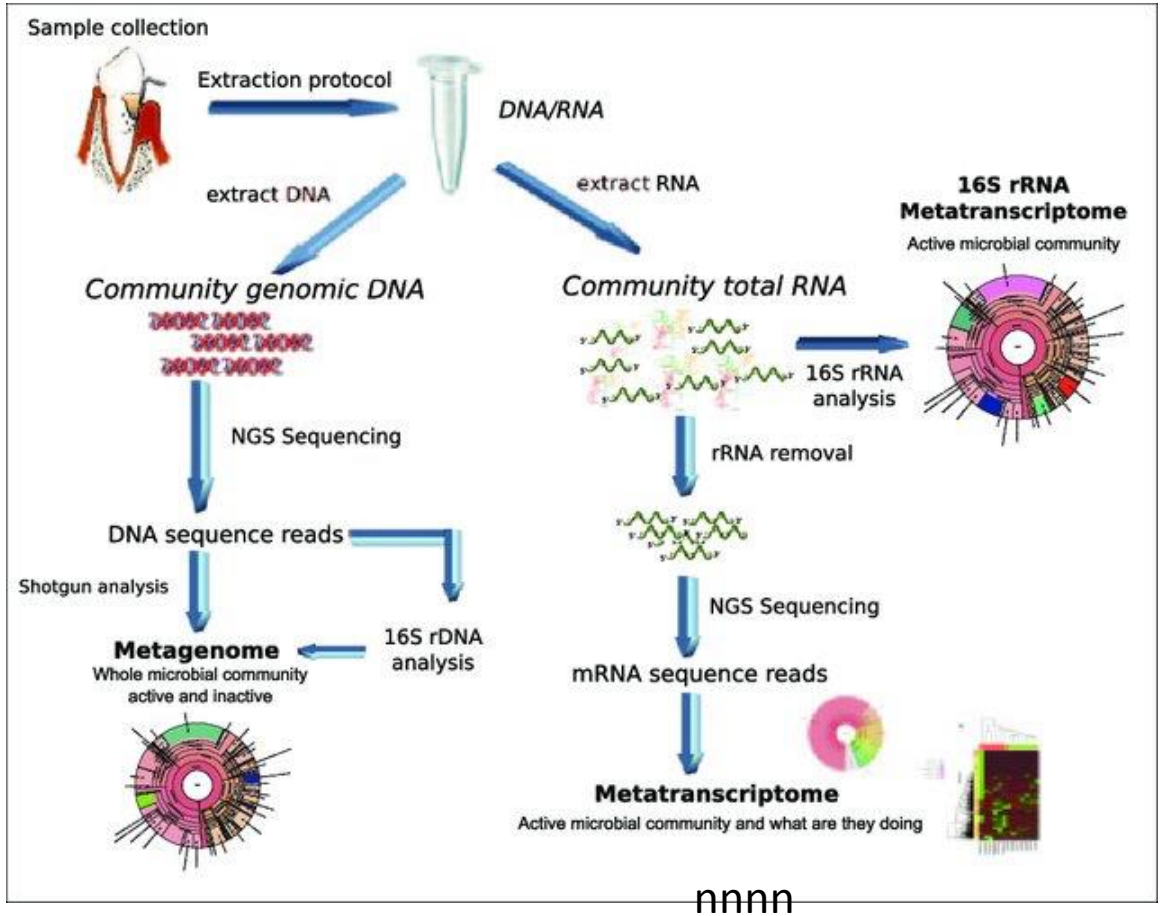


- Konak DNA kontaminasyonu
  - Filtreleme
- Yüksek Taksonomik ayırım gücü= Büyük Data
  - Biyoinformatik araçlar
- Tüm mikroorganizmaların tam genomlarının veri bankalarında olmaması
- Çok büyük sekans=Pahalı

Comparison of 16S rRNA gene sequencing (16S), metagenomic shotgun sequencing (MSS), and bacterial group quantitative polymerase chain reaction (qPCR).

	Time required for data production	Potential PCR bias	Microbial populations analyzed	Method of quantification	Functional analysis	Computing requirements	Data storage (per sample)	Estimated cost (excluding labor)
16S	Days to weeks	Yes	Bacteria only	Relative abundance	No	Open-source software that can be run on desktop computers	Megabytes	$10–$50/sample (if batched with a large number of samples)
MSS	Weeks to months	No	Bacteria, viruses, fungi	Relative abundance	Yes	Bioinformatic analysis is CPU intensive (requiring computer clusters)	Gigabytes	$200–$800/sample (depending on depth of coverage desired)

Metatranskriptomik Çalışmalar



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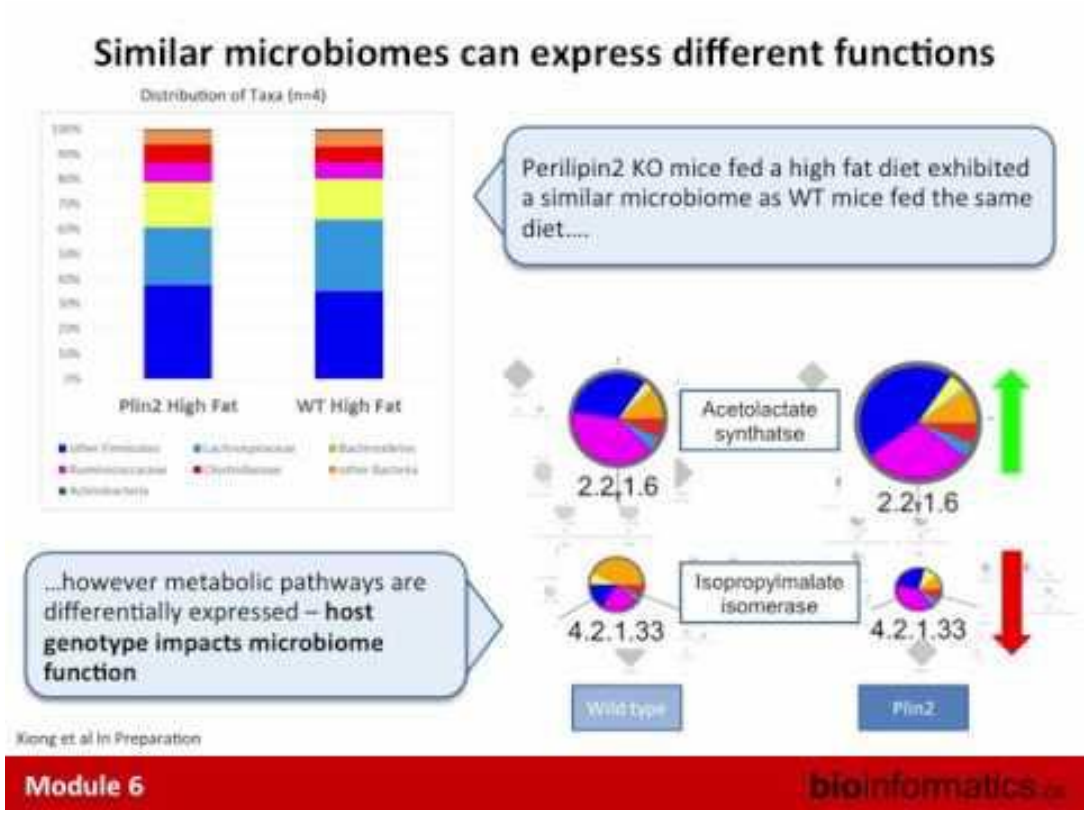
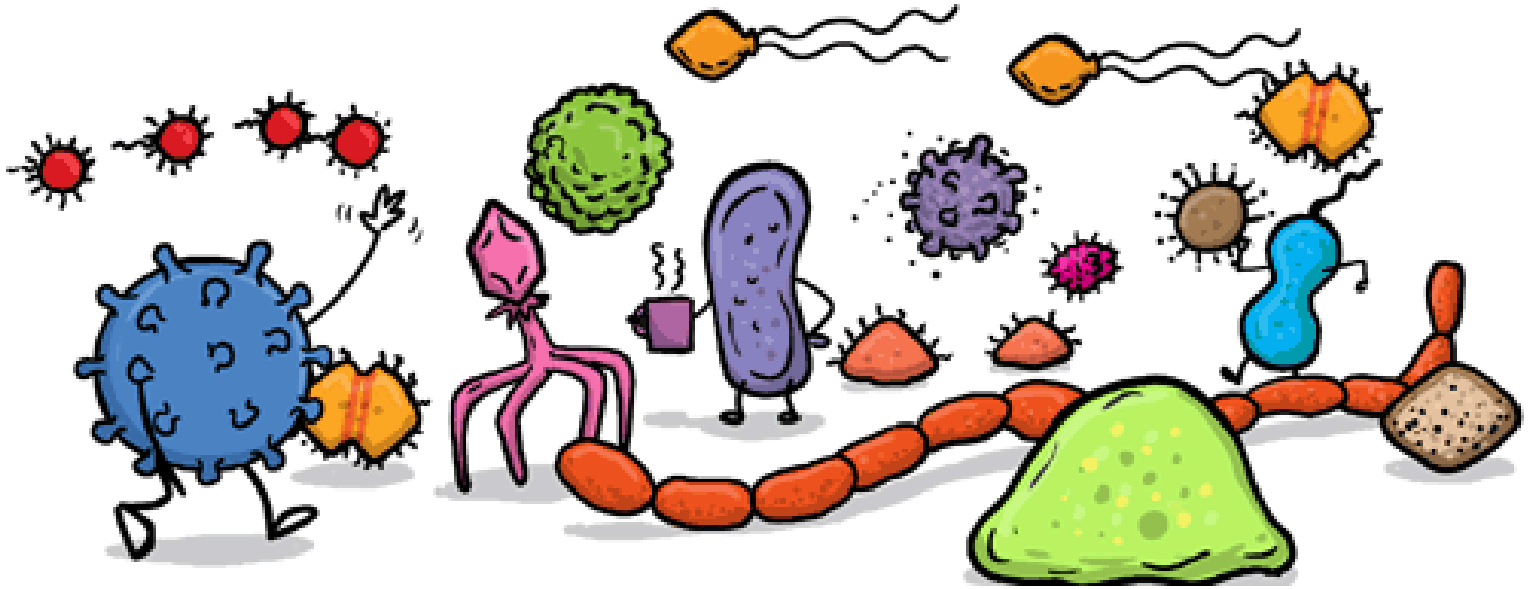


The Hidden World Of MICROBIOMES



Sayın Katılımcılar

Çalışma Grubumuzun Aktiviteleri için

Lütfen Hatta Kalınız….