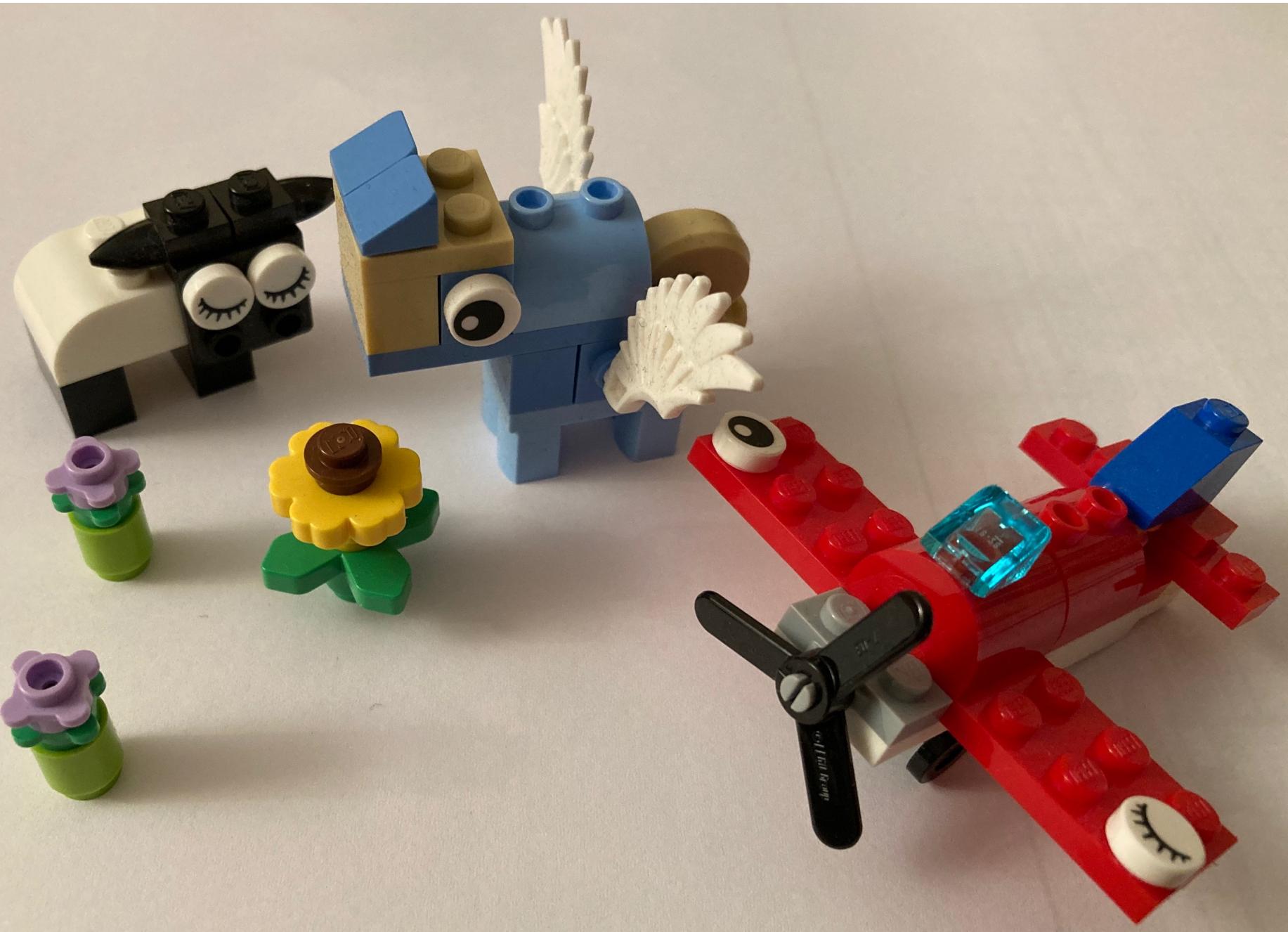




Advanced Applications of Next Generation Sequencing in Food Safety



Dr Guerrino Macori, BSc, MSc, PhD
Assistant Professor
School of Biology and Environmental Science
University College Dublin, Ireland
UCD Centre for Food Safety
guerrino.macori@ucd.ie
@guerrinomacori



Overview

- What is metagenomics
- Difference between 16s sequencing and shot-gun metagenomics
- Application of metagenomics
- The human and food microbiome
- What is metatranscriptomic

Microbiomes and metagenomes: Introduction to metagenomics

1. Background and introduction to metagenomics
2. 16s ribosomal RNA gene sequencing
3. Shotgun metagenomic sequencing
4. Application metagenomics food safety and food quality
5. Resistome
6. Food microbiome
7. Human microbiome
8. The microbiome of fermented foods, can we hack our gut microbiome?

Background

Microbiology approaches



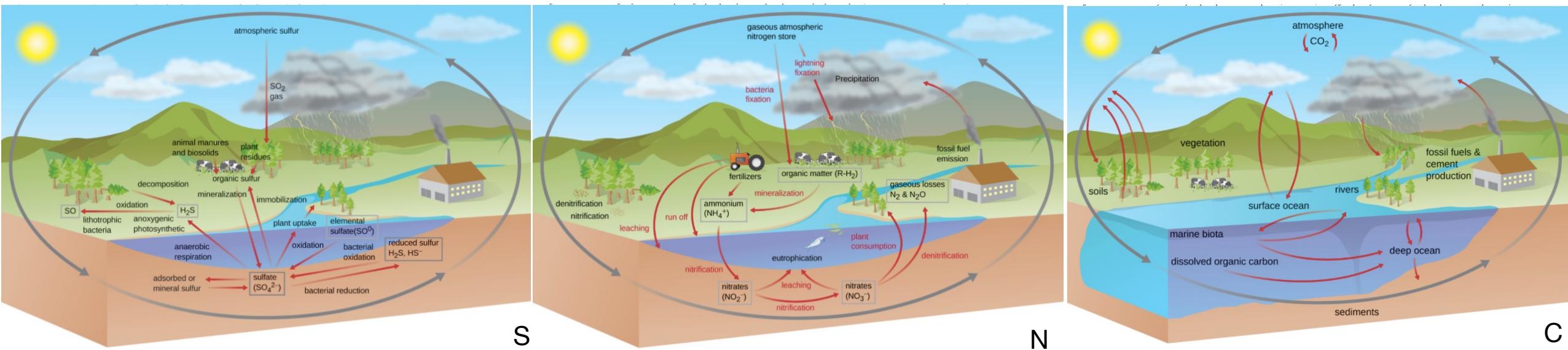
Culture-dependent methods

Early Microbiology and the Microscope



Background

Microbiology approaches



microorganisms are responsible of most of the geochemical cycles

Pictures: modification of work by NOAA – biogeochemical cycles
<https://ecampusontario.pressbooks.pub/microbio/chapter/biogeochemical-cycles/>

Background

Microbiology approaches

Neurobiology of Stress 7 (2017) 124–136

Contents lists available at ScienceDirect

Neurobiology of Stress

ELSEVIER journal homepage: <http://www.journals.elsevier.com/neurobiology-of-stress/>



Stress & the gut-brain axis: Regulation by the microbiome

Jane A. Foster ^a, Linda Rinaman ^{b,*}, John F. Cryan ^{c, d}

^a Department of Psychiatry & Behavioural Neurosciences, McMaster University, Hamilton, Ontario, Canada
^b Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, United States
^c APC Microbiome Institute, University College Cork, Cork, Ireland
^d Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

ARTICLE INFO

Article history:
Received 15 December 2016
Received in revised form
16 February 2017
Accepted 2 March 2017
Available online 19 March 2017

ABSTRACT

The importance of the gut-brain axis in regulating stress-related responses has long been appreciated. More recently, the microbiota has emerged as a key player in the control of this axis, especially during conditions of stress provoked by real or perceived homeostatic challenge. Diet is one of the most important modifying factors of the microbiota-gut-brain axis. The routes of communication between the microbiota and brain are slowly being unravelled, and include the vagus nerve, gut hormone signalling, the immune system, tryptophan metabolism, and microbial metabolites such as short chain fatty acids. The importance of the early life gut microbiota in shaping later health outcomes also is emerging. Results from preclinical studies indicate that alterations of the early microbial composition by way of antibiotic exposure, lack of breastfeeding, birth by Caesarean section, infection, stress exposure, and other environmental influences - coupled with the influence of host genetics - can result in long-term modulation of stress-related physiology and behaviour. The gut microbiota has been implicated in a variety of stress-related conditions including anxiety, depression and irritable bowel syndrome, although this is largely based on animal studies or correlative analysis in patient populations. Additional research in humans is sorely needed to reveal the relative impact and causal contribution of the microbiome to stress-related disorders. In this regard, the concept of psychobiotics is being developed and refined to encompass methods of targeting the microbiota in order to positively impact mental health outcomes. At the 2016 Neurobiology of Stress Workshop in Newport Beach, CA, a group of experts presented the symposium "The Microbiome: Development, Stress, and Disease". This report summarizes and builds upon some of the key concepts in that symposium within the context of how microbiota might influence the neurobiology of stress.

© 2017 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Foster et al., 2016

nature
microbiology

ARTICLES

<https://doi.org/10.1038/s41564-018-0337-x>

The neuroactive potential of the human gut microbiota in quality of life and depression

Mireia Valles-Colomer ^{1,2}, Gwen Falony ^{1,2}, Youssef Darzi ^{1,2}, Ettje F. Tigchelaar ³, Jun Wang ^{1,2}, Raul Y. Tito ^{1,2,4}, Carmen Schiweck ⁵, Alexander Kurnilshikov ^{1,2}, Marie Joossens ^{1,2}, Cisca Wijmenga ^{1,2,6}, Stephan Claes ^{5,7}, Lukas Van Oudenhove ^{7,8}, Alexandra Zhernakova ³, Sara Vieira-Silva ^{1,2,9} and Jeroen Raes ^{1,2,9*}

The relationship between gut microbial metabolism and mental health is one of the most intriguing and controversial topics in microbiome research. Bidirectional microbiota-gut-brain communication has mostly been explored in animal models, with human research lagging behind. Large-scale metagenomics studies could facilitate the translational process, but their interpretation is hampered by a lack of dedicated reference databases and tools to study the microbial neuroactive potential. Surveying a large microbiome population cohort (Flemish Gut Flora Project, $n = 1,054$) with validation in independent data sets ($n_{\text{total}} = 1,070$), we studied how microbiome features correlate with host quality of life and depression. Butyrate-producing *Faecalibacterium* and *Coprococcus* bacteria were consistently associated with higher quality of life indicators. Together with *Dialister*, *Coprococcus* spp. were also depleted in depression, even after correcting for the confounding effects of antidepressants. Using a module-based analytical framework, we assembled a catalogue of neuroactive potential of sequenced gut prokaryotes. Gut-brain module analysis of faecal metagenomes identified the microbial synthesis potential of the dopamine metabolite 3,4-dihydroxyphenylacetic acid as correlating positively with mental quality of life and indicated a potential role of microbial γ -aminobutyric acid production in depression. Our results provide population-scale evidence for microbiome links to mental health, while emphasizing confounder importance.

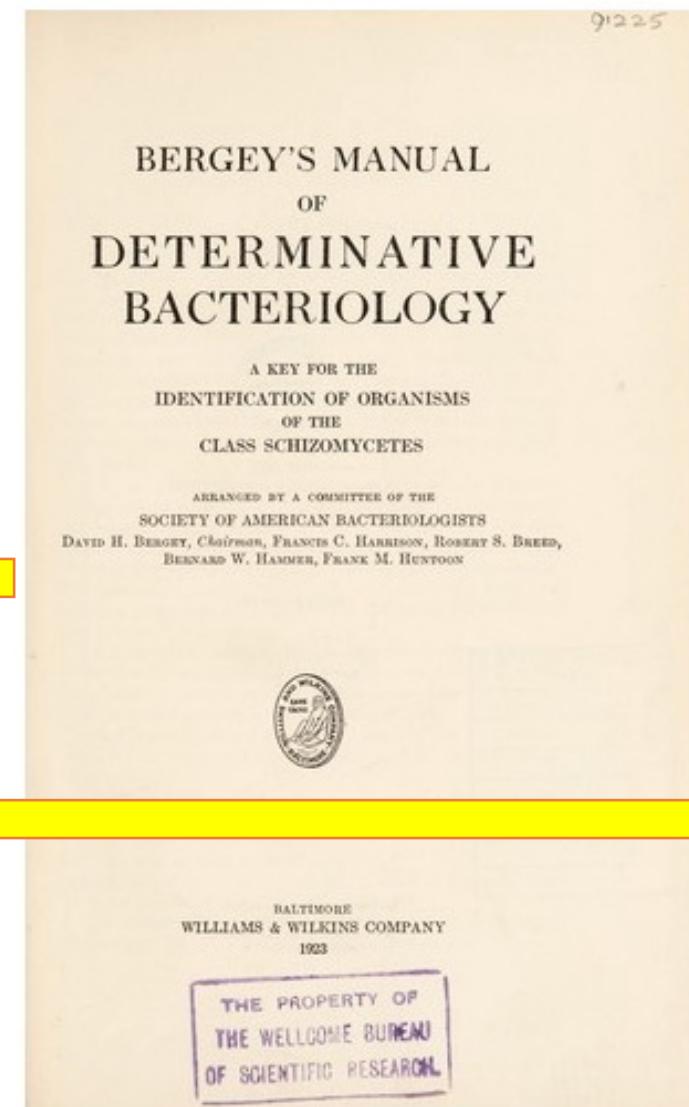
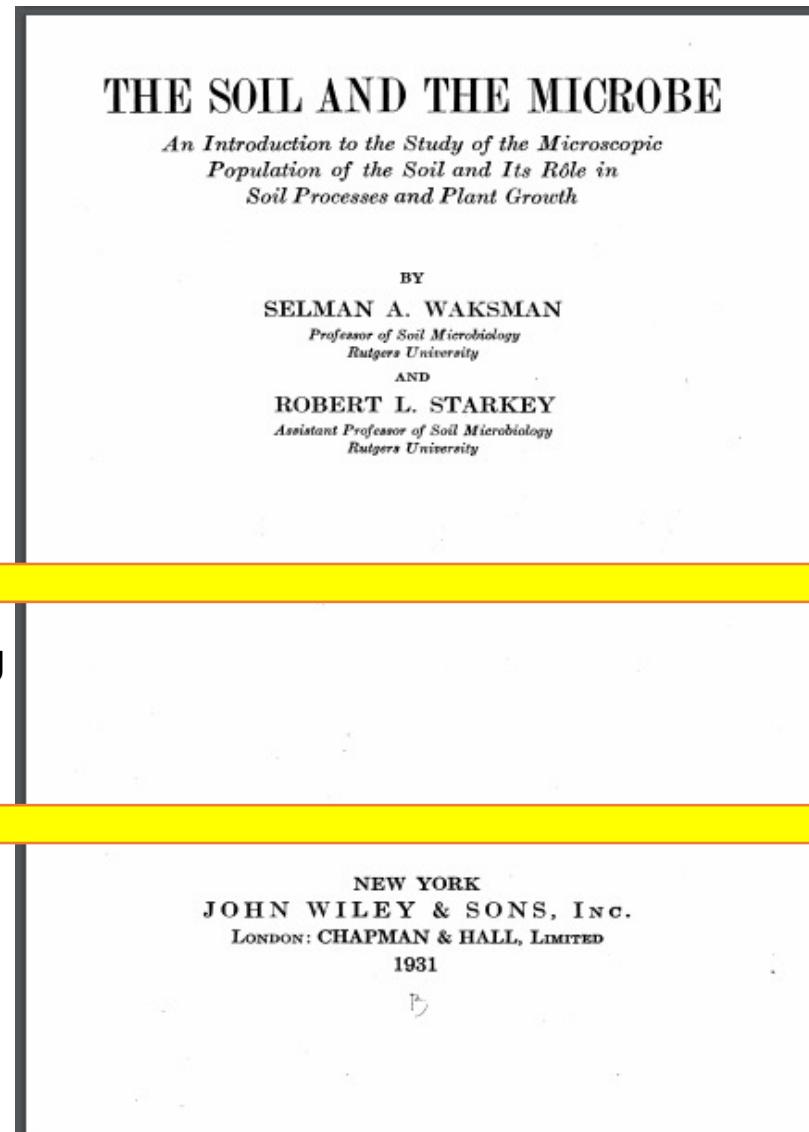
Valles-Colomer et al., 2019

Background

Microbiology approaches

“knowledge of the soil microbes, enables us to construct a clear picture”
Waksman & Starkey, 1931

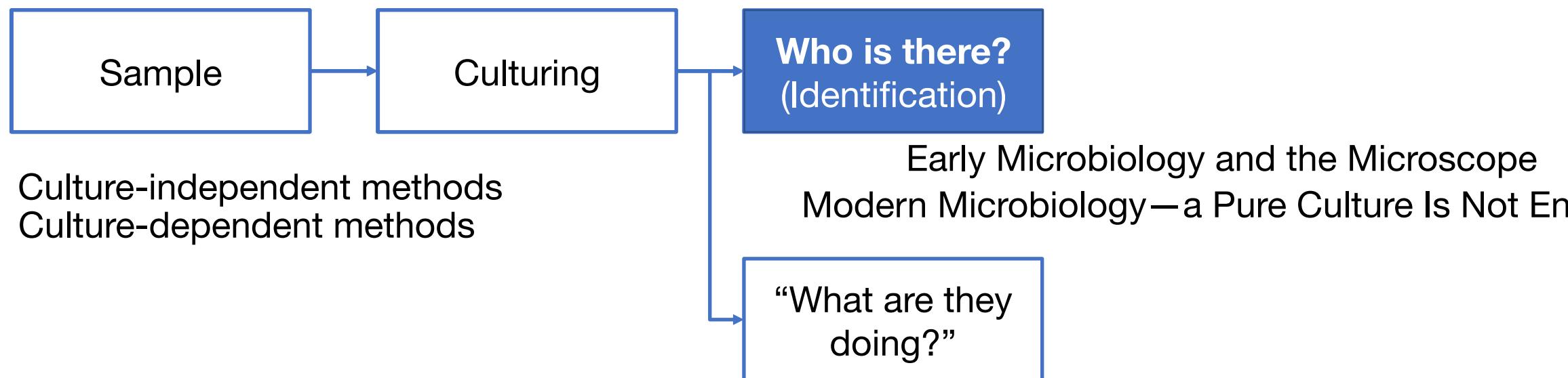
“No organism could be classified without being cultured ”
Berger's Manual of bacteriology, 1923



Background

Microbiology approaches

Classification, identification



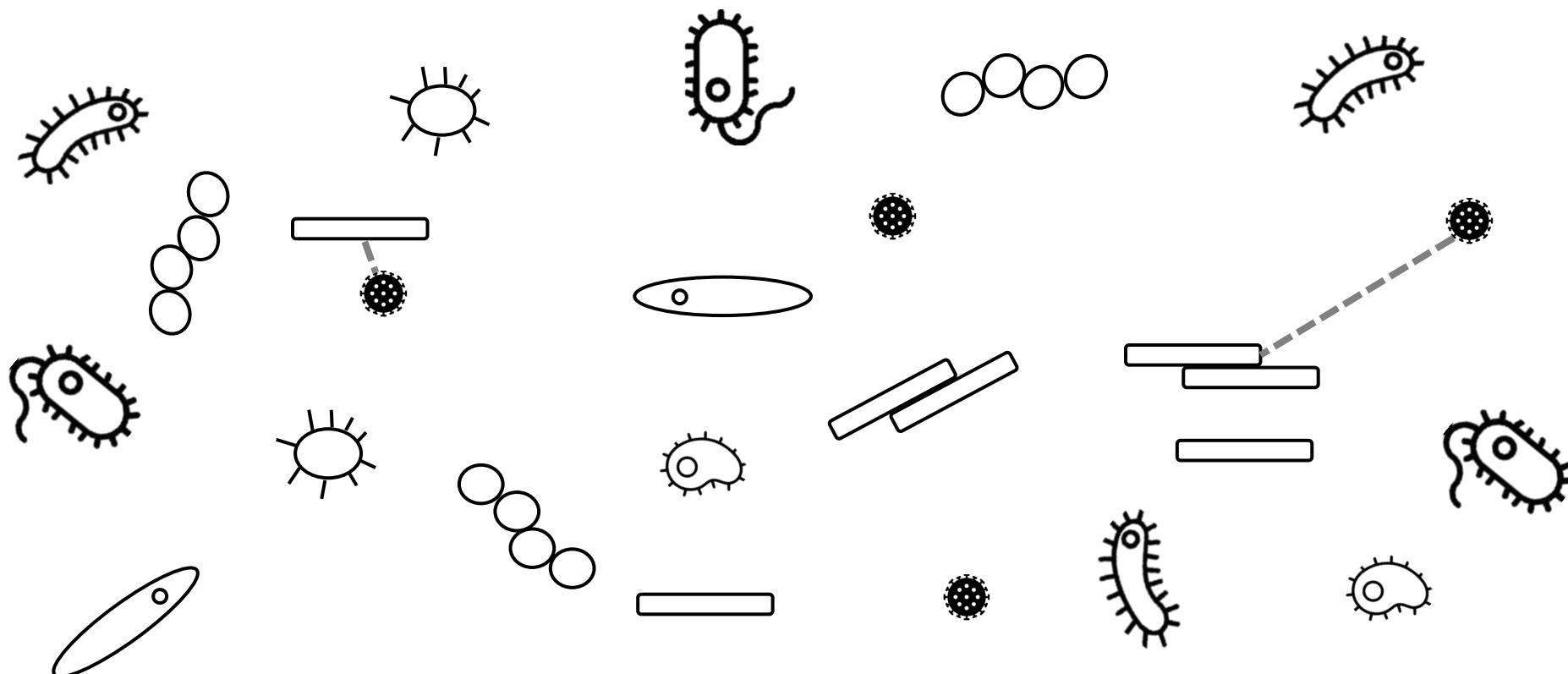
1. Introduction

“Metagenomics is a molecular tool used to analyse DNA acquired from samples, in order to study the community of microorganisms present, without the necessity of obtaining pure cultures.”

Ghosh et al., 2019

1. Introduction

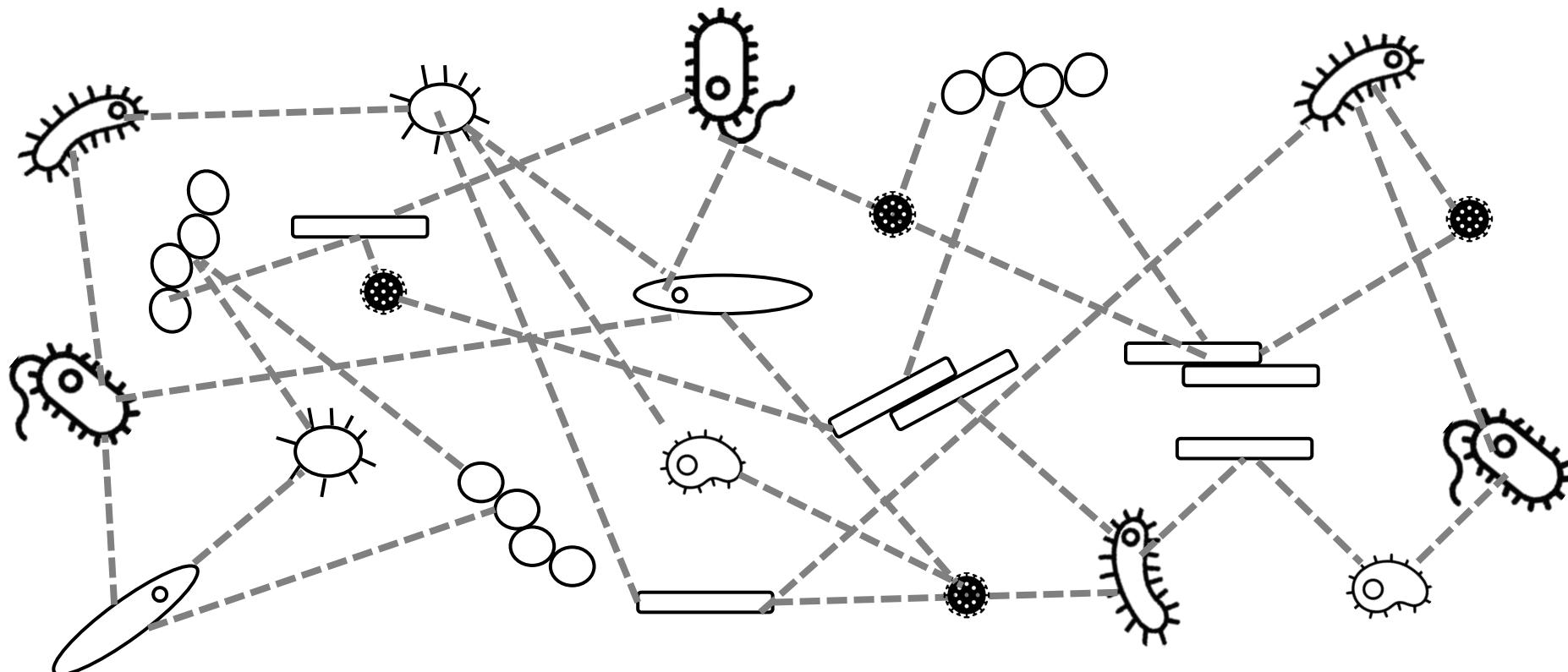
A microbiota is the community of microorganisms living together in a particular habitat



Adapted from "The microbiome explained". Video by the Microbiology Society

1. Introduction

A microbiome is the community of microorganisms living together in a particular habitat in a complex microsystem of interlinked networks and functions: includes microorganisms and their genes.



Adapted from "The microbiome explained". Video by the Microbiology Society

1. Introduction

The phylogeny of prokaryotes

Searching for similarity: from observation to genomics

| Kingdom | When Evolved | Structure | Photosynthesis |
|---------------------|--------------------------|------------------------------|----------------|
| Prokaryotic: | | | |
| Bacteria | 3 to 4 billion years ago | Unicellular | Sometimes |
| Archaea | 3 to 4 billion years ago | Unicellular | No |
| Eukaryotic: | | | |
| Protista | 1.5 billion years ago | Unicellular | Sometimes |
| Fungi | 1 billion years ago | Unicellular or Multicellular | No |
| Animalia | 700 million years ago | Multicellular | No |
| Plantae | 500 million years ago | Multicellular | Yes |

J. molec. Evolution 2, 99—116 (1973)
© by Springer-Verlag 1973

Eukaryote Evolution: A View Based on Cytochrome c Sequence Data

P. J. McLaughlin and M. O. Dayhoff
National Biomedical Research Foundation,
Georgetown University Medical Center, Washington, D.C.
Received March 19, 1973

Summary. We have compared the amino acid sequences of cytochrome c's from 45 species of organisms representing all five kingdoms, including one species each for the Protista and Monera. We have made a phylogeny for these data by reconstructing probable ancestral sequences which generate the present descendants through a minimum number of mutations. Several trials with different data sets produced the same minimal configuration. Assuming the occurrence of no major shifts in mutation acceptance rate, we find an early differentiation between prokaryote and eukaryote stocks. Afterward the eukaryote stem gave rise first to the protozoan flagellate branch and later to the multicellular green plant branch; after this the fungi and multicellular animal stems diverged from each other. A probable ancestral sequence was estimated for each kingdom of multicellular organisms. The basic eukaryote ancestor was probably a non-photosynthetic, heterotrophic flagellate. The photosynthetic apparatus could have been a later symbiotic acquisition in the plant ancestry. The dicotyledons had differentiated into two stocks before the emergence of a monocotyledon line as did the Ascomycetes before the emergence of the Basidiomycetes. The mollusc and chordate lines may have had a common acelomate ancestor at the divergence of the arthropod stock. The numbers of mutations on all of the branches of the phylogenetic tree were calculated as well as the numbers of mutations and repeated mutations at each amino acid position.

Key words: Eukaryote Phylogeny — Cytochrome c — Evolutionary Tree — Ancestral Sequences — Mutations.

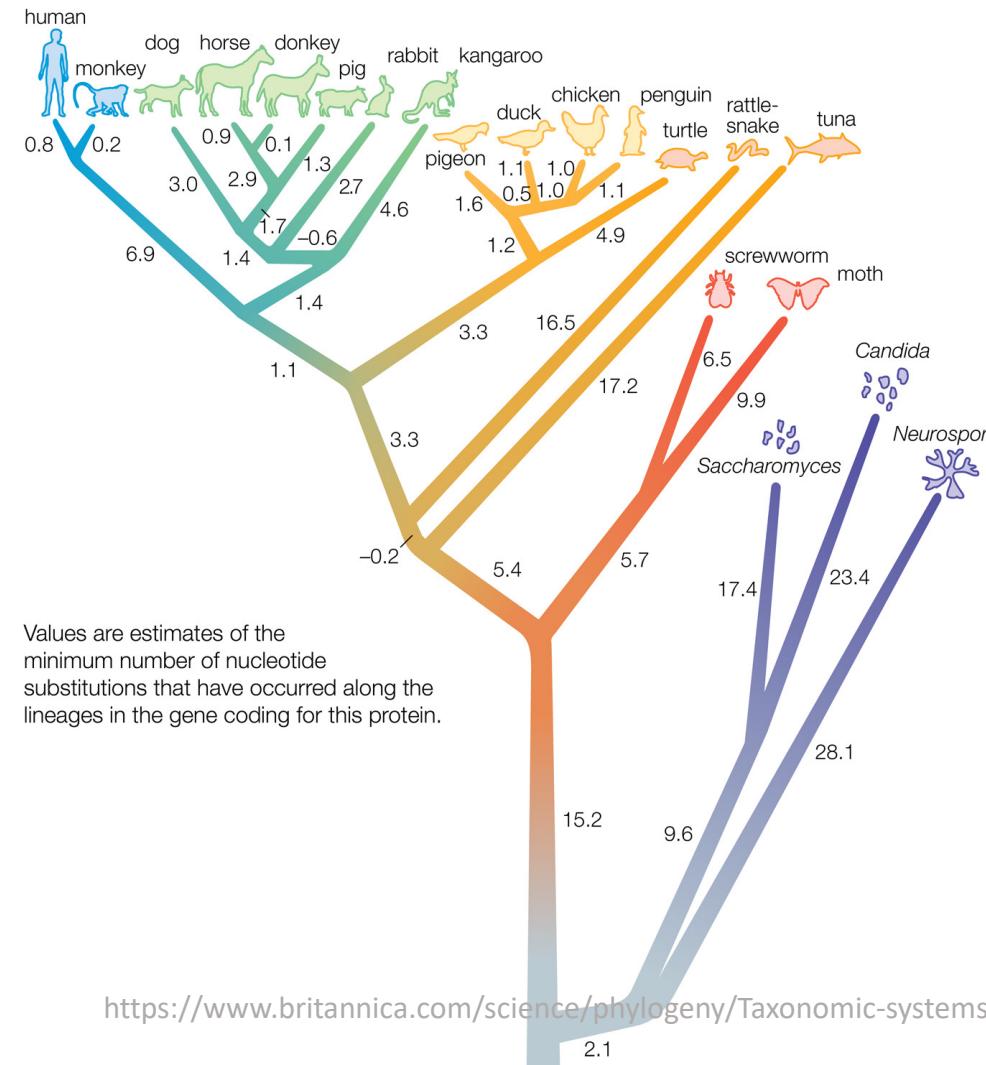
Introduction

Our knowledge of evolution is greatly increased by several new sequences of cytochrome c, especially the first one from a unicellular organism (Pettigrew, 1972). This protistan is *Critchidia oncopelti*, a trypanosomatid flagellate parasitic in insects. *Critchidia* has a functional mitochondrial cytochrome system containing a cytochrome c comparable with those of multicellular organisms. The crithidial protein contains trimethyllysine, as do proteins from green plants and fungi; it reacts with mammalian cytochrome oxidase, although at a reduced rate (Hill, Chan, and Smith, 1971); and the sequence is homologous with other cytochrome c sequences. Cytochrome c sequences are now known for representatives of the five kingdoms of organisms—the Fungi, the multicellular green plants (Plantae), the multicellular animals

1. Introduction

The phylogeny of prokaryotes

Searching for similarity: from observation to genomics



J. molec. Evolution 2, 99–116 (1973)
© by Springer-Verlag 1973

Eukaryote Evolution: A View Based on Cytochrome c Sequence Data

P. J. McLaughlin and M. O. Dayhoff
National Biomedical Research Foundation,
Georgetown University Medical Center, Washington, D.C.

Received March 19, 1973

Summary. We have compared the amino acid sequences of cytochrome *c*'s from 45 species of organisms representing all five kingdoms, including one species each for the Protista and Monera. We have made a phylogeny for these data by reconstructing probable ancestral sequences which generate the present descendants through a minimum number of mutations. Several trials with different data sets produced the same minimal configuration. Assuming the occurrence of no major shifts in mutation acceptance rate, we find an early differentiation between prokaryote and eukaryote stocks. Afterward the eukaryote stem gave rise first to the protozoan flagellate branch and later to the multicellular green plant branch; after this the fungi and multicellular animal stems diverged from each other. A probable ancestral sequence was estimated for each kingdom of multicellular organisms. The basic eukaryote ancestor was probably a non-photosynthetic, heterotrophic flagellate. The photosynthetic apparatus could have been a later symbiotic acquisition in the plant ancestry. The dicotyledons had differentiated into two stocks before the emergence of a monocotyledon line as did the Ascomycetes before the emergence of the Basidiomycetes. The mollusc and chordate lines may have had a common acelomate ancestor at the divergence of the arthropod stock. The numbers of mutations on all of the branches of the phylogenetic tree were calculated as well as the numbers of mutations and repeated mutations at each amino acid position.

Key words: Eukaryote Phylogeny — Cytochrome *c* — Evolutionary Tree — Ancestral Sequences — Mutations.

Introduction

Our knowledge of evolution is greatly increased by several new sequences of cytochrome *c*, especially the first one from a unicellular organism (Pettigrew, 1972). This protistan is *Critchidia oncopelti*, a trypanosomatid flagellate parasitic in insects. *Critchidia* has a functional mitochondrial cytochrome system containing a cytochrome *c* comparable with those of multicellular organisms. The crithidial protein contains trimethyllysine, as do proteins from green plants and fungi; it reacts with mammalian cytochrome oxidase, although at a reduced rate (Hill, Chan, and Smith, 1971); and the sequence is homologous with other cytochrome *c* sequences. Cytochrome *c* sequences are now known for representatives of the five kingdoms of organisms—the Fungi, the multicellular green plants (Plantae), the multicellular animals

1. Introduction

The phylogeny of prokaryotes

Searching for similarity: from observation to genomics

1970: cytochrome C sequences used for establishing eukaryotic phylogenies

Bacterial genealogies are more ancient

Comparative analysis of the **16S ribosomal RNA** sequence used to explore prokaryote phylogeny

- Universally distributed
- Constancy of function
- Change in sequence slowly

1980: 170 individual species were characterised using a sequencing approach producing a catalogue of sequences characteristic of the organism

J. molec. Evolution 2, 99–116 (1973)
© by Springer-Verlag 1973

**Eukaryote Evolution:
A View Based on Cytochrome c
Sequence Data**

P. J. McLaughlin and M. O. Dayhoff
National Biomedical Research Foundation,
Georgetown University Medical Center, Washington, D.C.

Received March 19, 1973

Summary. We have compared the amino acid sequences of cytochrome *c*'s from 45 species of organisms representing all five kingdoms, including one species each for the Protista and Monera. We have made a phylogeny for these data by reconstructing probable ancestral sequences which generate the present descendants through a minimum number of mutations. Several trials with different data sets produced the same minimal configuration. Assuming the occurrence of no major shifts in mutation acceptance rate, we find an early differentiation between prokaryote and eukaryote stocks. Afterward the eukaryote stem gave rise first to the protozoan flagellate branch and then to the multicellular green plant branch; after this the fungi and multicellular animal stems diverged from each other. A probable ancestral stock was estimated for each kingdom of multicellular organisms. The basic eukaryote ancestor was probably a non-photosynthetic, heterotrophic flagellate. The photosynthetic apparatus could have been a later symbiotic acquisition in the plant ancestry. The dicotyledons had differentiated into two stocks before the emergence of a monocotyledon line as did the Ascomycetes before the emergence of the Basidiomycetes. The mollusc and chordate lines may have had a common acelomate ancestor at the divergence of the arthropod stock. The numbers of mutations on all of the branches of the phylogenetic tree were calculated as well as the numbers of mutations and repeated mutations at each amino acid position.

Key words: Eukaryote Phylogeny — Cytochrome *c* — Evolutionary Tree — Ancestral Sequences — Mutations.

Introduction

Our knowledge of evolution is greatly increased by several new sequences of cytochrome *c*, especially the first one from a unicellular organism (Pettigrew, 1972). This protistan is *Criithidia oncopelti*, a trypanosomatid flagellate parasitic in insects. *Criithidia* has a functional mitochondrial cytochrome system containing a cytochrome *c* comparable with those of multicellular organisms. The crithidial protein contains trimethyllysine, as do proteins from green plants and fungi; it reacts with mammalian cytochrome oxidase, although at a reduced rate (Hill, Chan, and Smith, 1971); and the sequence is homologous with other cytochrome *c* sequences. Cytochrome *c* sequences are now known for representatives of the five kingdoms of organisms—the Fungi, the multicellular green plants (Plantae), the multicellular animals

McLaughlin & Dayhoff, 1973

Reprint Series
25 July 1980, Volume 209, pp. 457–463

SCIENCE

The Phylogeny of Prokaryotes

G. E. Fox, E. Stackebrandt, R. B. Hespell, J. Gibson
J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch
R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore
R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl
K. R. Luehrs, K. N. Chen, C. R. Woese

Copyright © 1980 by the American Association for the Advancement of Science

Fox et al., 1980

1. Introduction

The phylogeny of prokaryotes

Searching for similarity: from observation to genomics

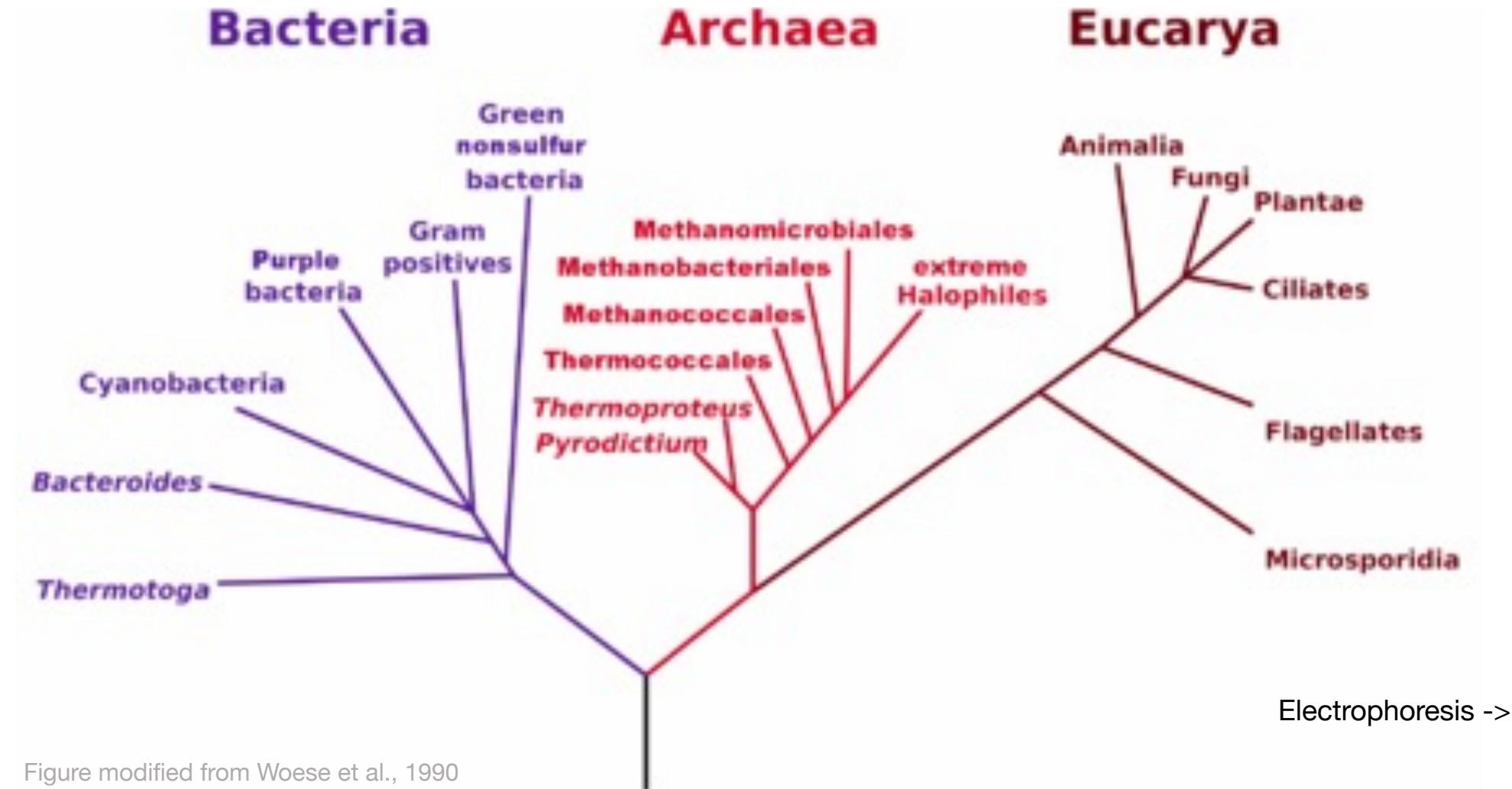


Figure modified from Woese et al., 1990

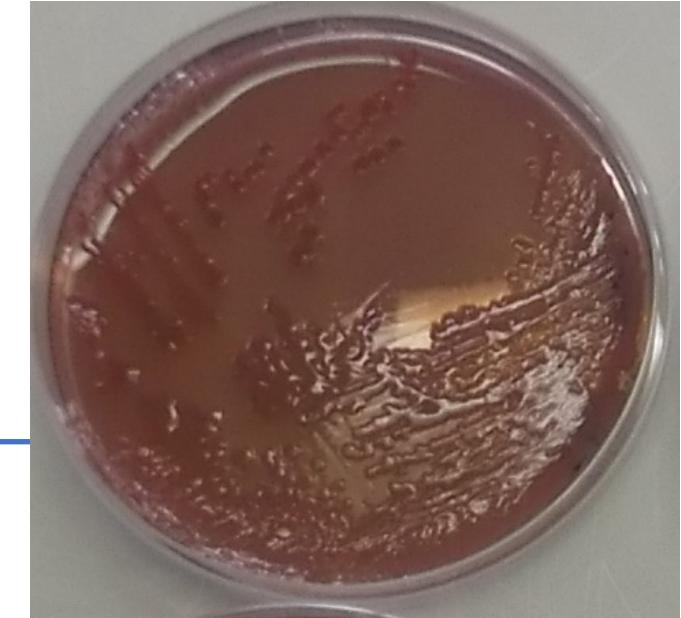
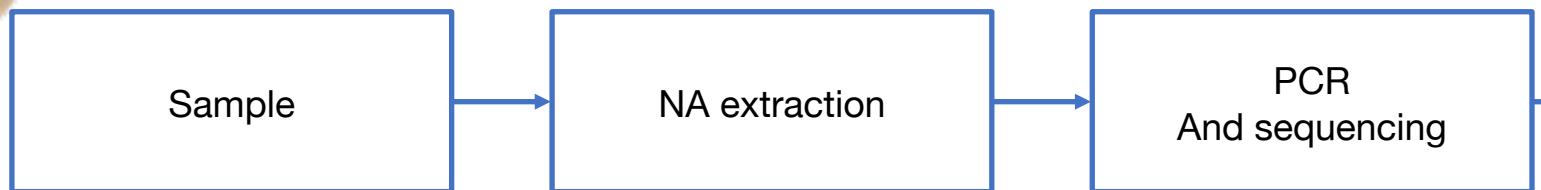
1. Introduction

The phylogeny of prokaryotes

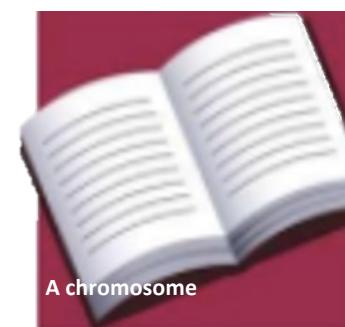
Searching for similarity: from observation to genomics



TGTAGAAAAAGGTACAGTAAAAAAATCCTAATCTAGGTTAATATAATTGAT
CAGAAGAAAATATTACAAATGTACAAGAAAATGCTTCAGATTATAACGAAGA
GTAAGAAAAAGACCCGGTATGTACATTGGTTCAACTAGCCAACGCAGGGCTCC
AATTGATGAAGGACTTCTGGTTTTGTACAGAAATTGAAATTACAATTGAA
GACGTGGATTTCCTACAGGGATTAATGAAAAAAATCGGTGTCACAGTAGA
AAATTGGCGGCCGGGATATAAGTATCTGGCGGACTTCACGGAGTTGGTC
TGAAGTATACTTCACCGTGAAGGTCAAAAATATTACCAACGCTTGAAACGT



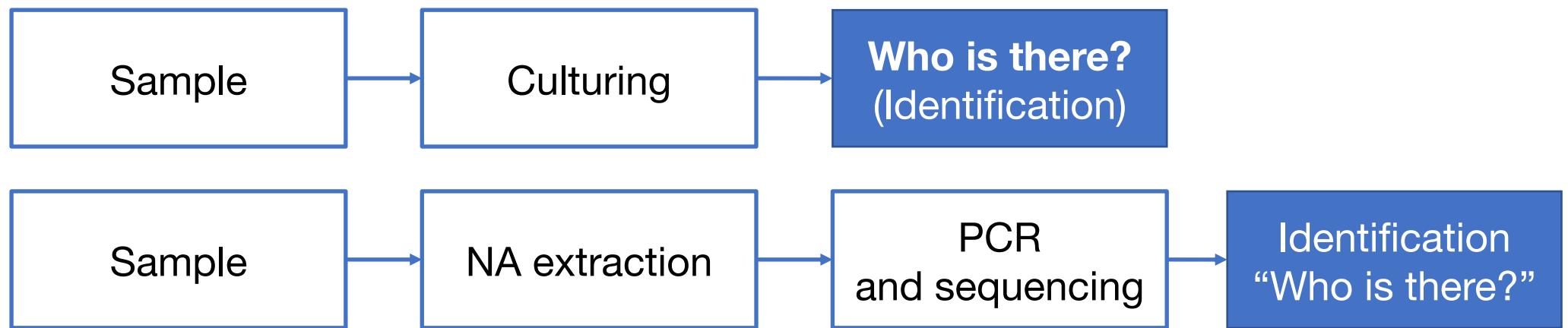
Cookbook analogy



1. Introduction

The phylogeny of prokaryotes

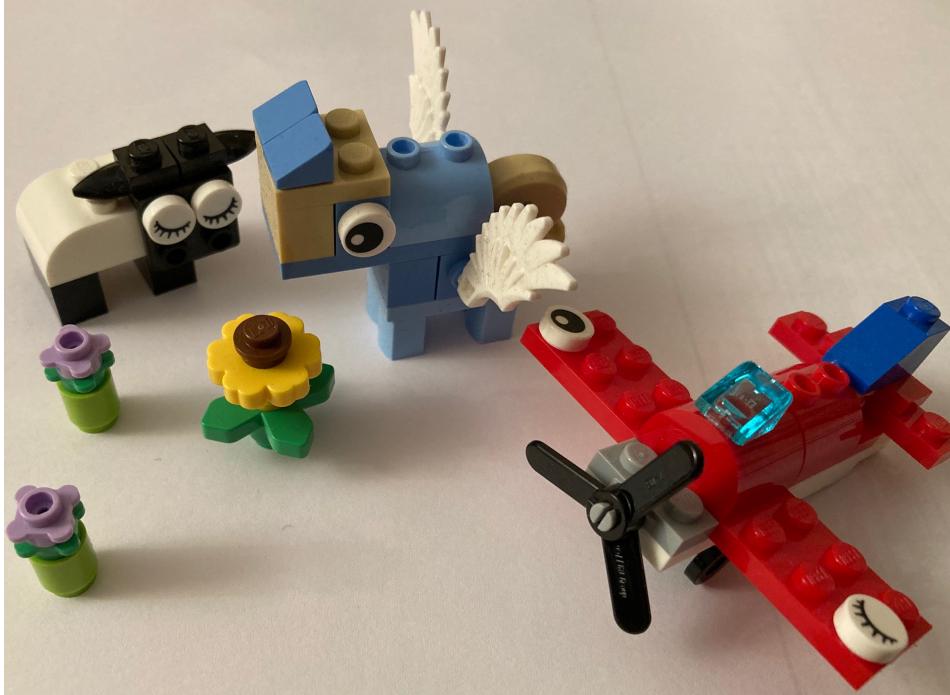
Searching for similarity: from observation to genomics



1. Introduction

The phylogeny of prokaryotes

Searching for similarity: from observation to genomics



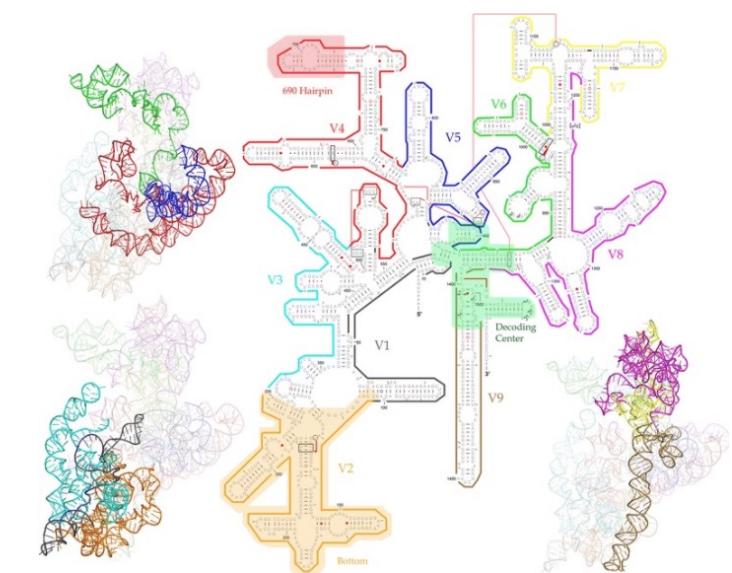
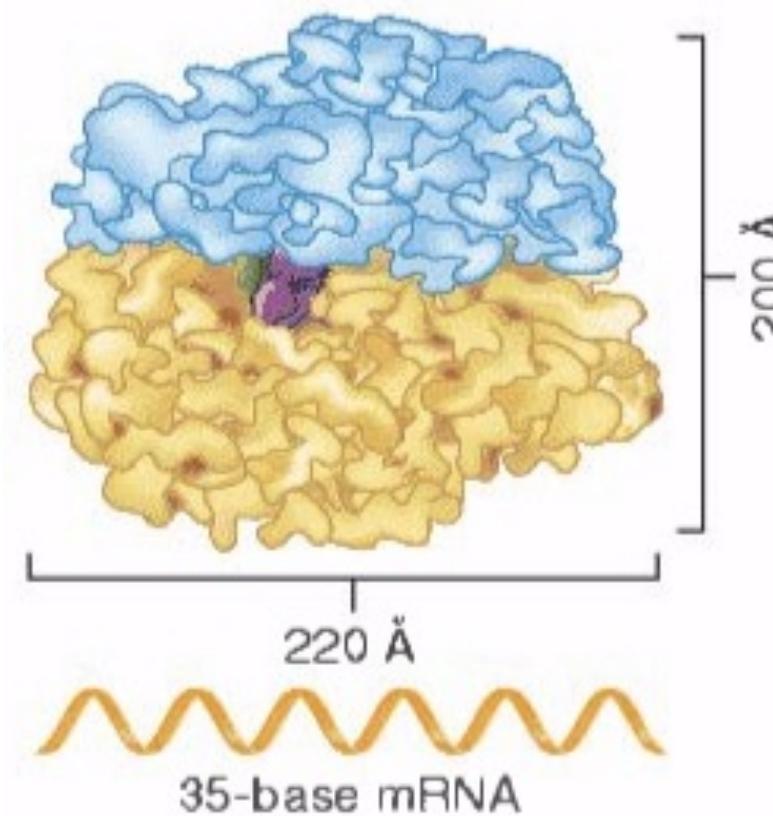
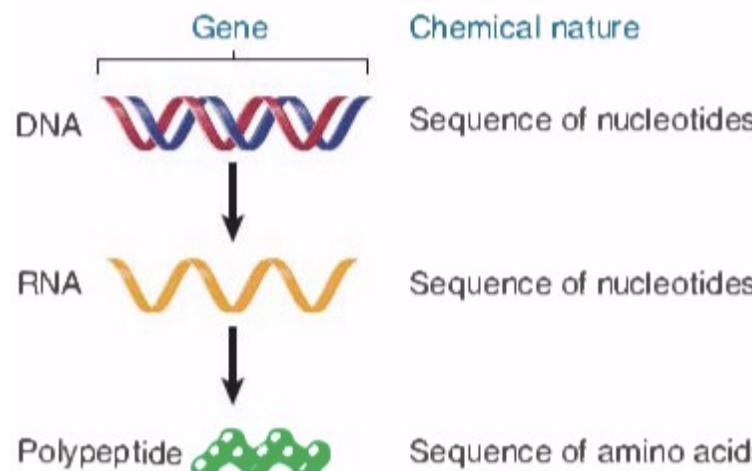
Study the different microbial objects in a community:

- All the DNA present
- Ubiquitous gene with some variable and conserved regions

1. Introduction

Ribosome and rRNA

Ribosomes are macromolecular machines, found within all living cells -> protein synthesis (mRNA translation)



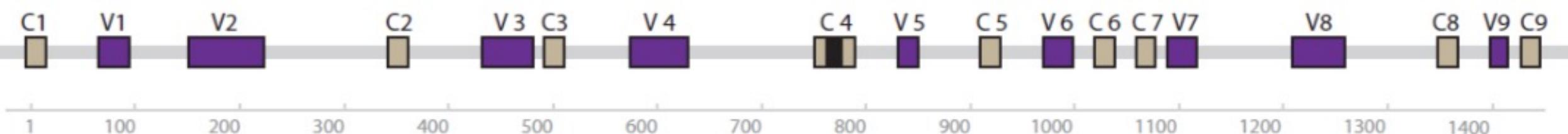
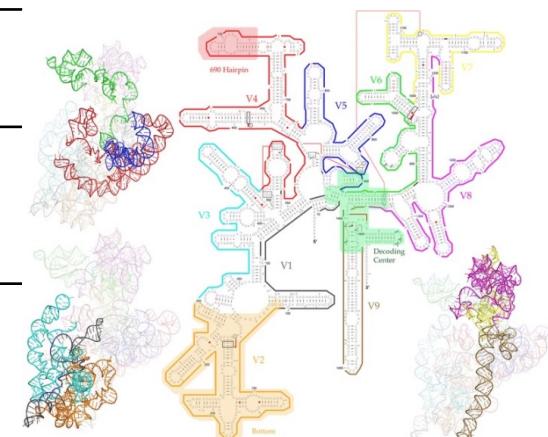
Pictures by Lewin' Genes XI

1. Introduction

Ribosome and rRNA

Ribosomes are macromolecular machines, found within all living cells -> protein synthesis (mRNA translation)

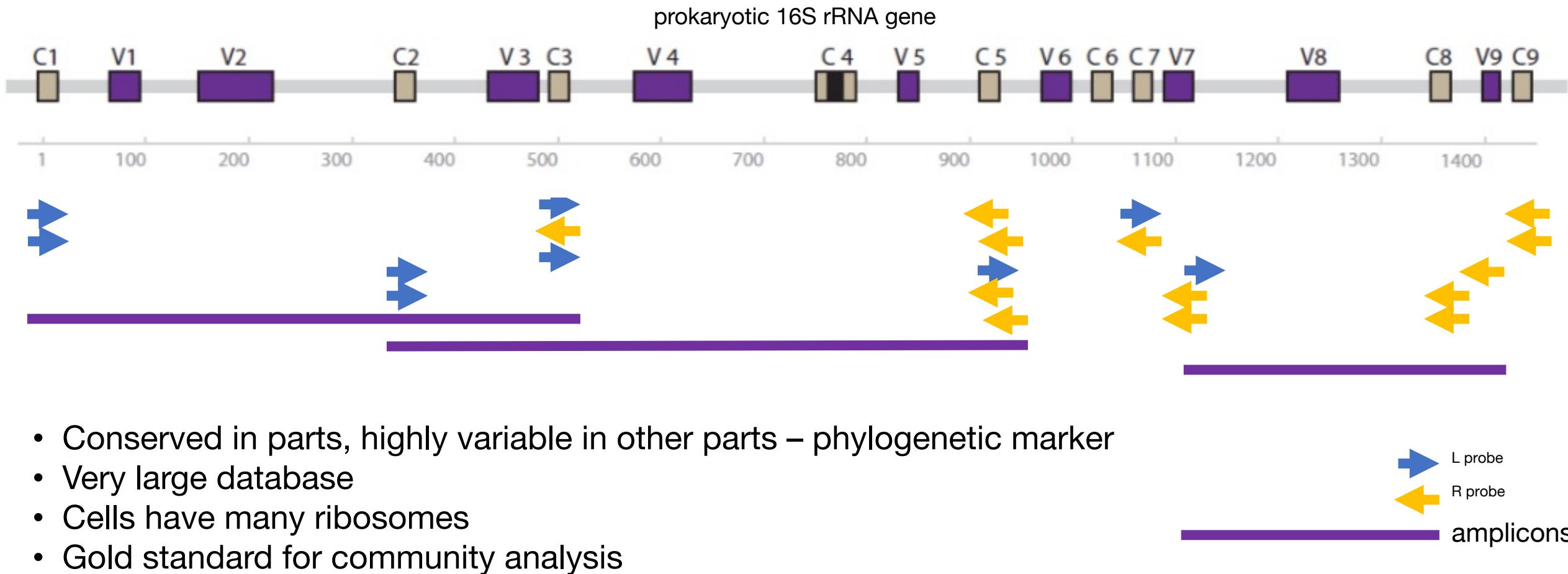
| Type | Size | Large subunit | Small subunit |
|-------------|------|---|--|
| prokaryotic | 70S | 50S rRNA 5S (120 nt) 23S (2904 nt) | 30S rRNA 16S (1542 nt) |
| eukaryotic | 80S | 60S rRNA 28S (4718 nt) 5.8S (160 nt) 5S (120 nt) | 40S rRNA 18S (1874 nt) |



Pictures by Lewin' Genes XI

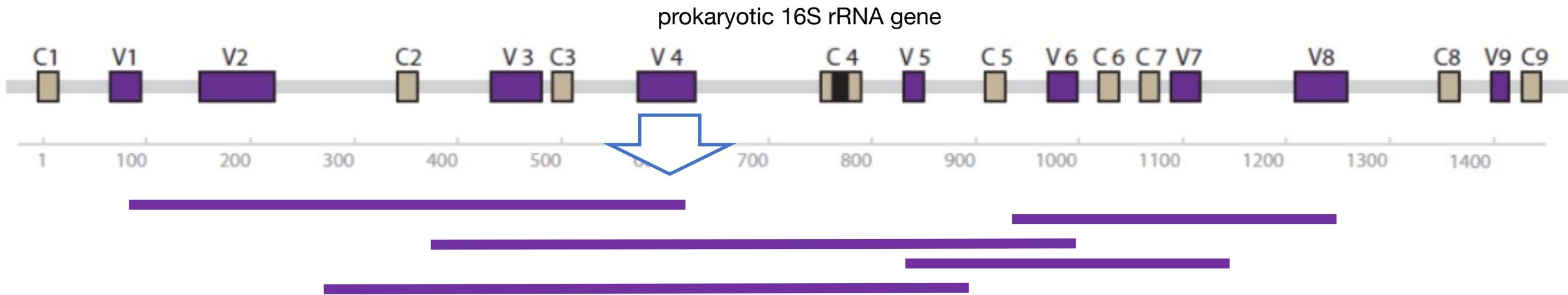
2. 16S rRNA gene sequencing

Ribosome and rRNA



2. 16S rRNA gene sequencing

Ribosome and rRNA



| Region | Position | bp |
|--------|-----------|-----|
| V1 | 69-99 | 30 |
| V2 | 137-242 | 105 |
| V3 | 338-533 | 195 |
| V4 | 576-682 | 106 |
| V5 | 822-879 | 57 |
| V6 | 967-1046 | 79 |
| V7 | 1117-1173 | 56 |
| V8 | 1243-1294 | 51 |
| V9 | 1435-1465 | 30 |

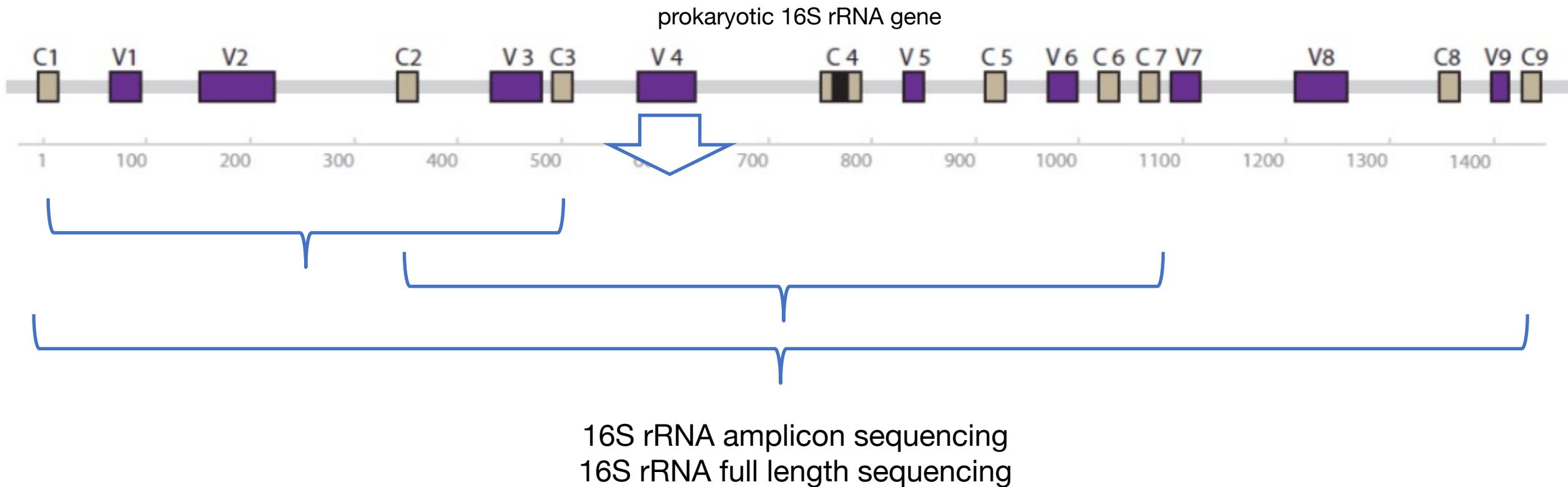
V4 Hypervariable region

Produces 288-290 bp amplicons

Can be sequenced via Illumina MiSeq chemistry 2x250 bp

2. 16S rRNA gene sequencing

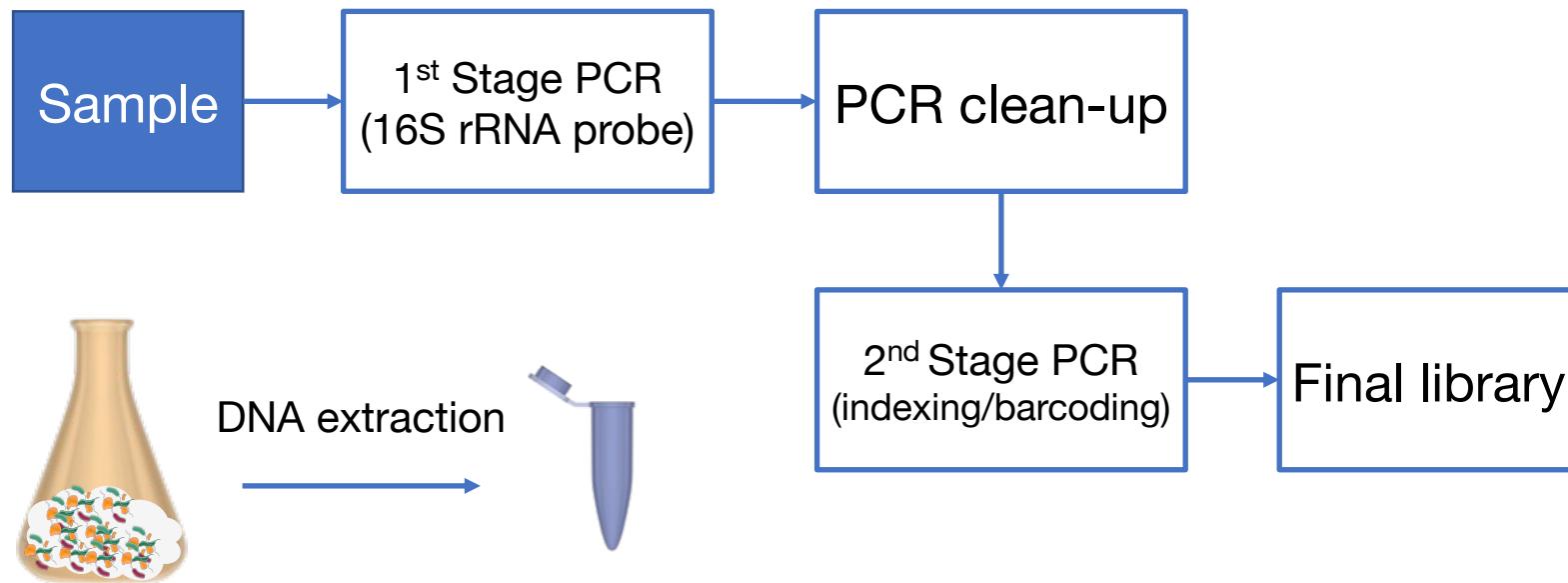
Ribosome and rRNA



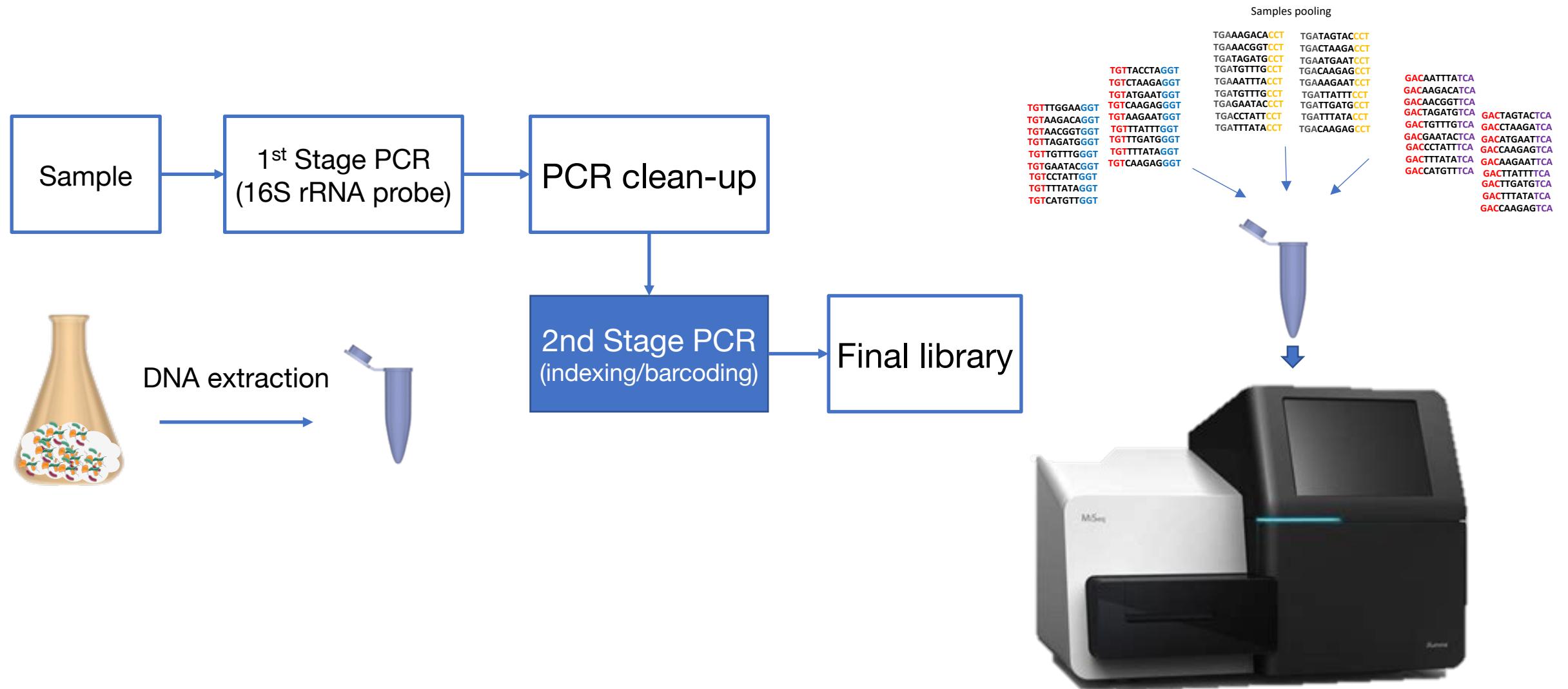
2. 16S rRNA gene sequencing

- Six Kingdoms: Eubacteria, Archaebacteria, Protista, Fungi, Plantae, Animalia
- Three domains of life: Bacteria, Archea, Eukarya
- **Taxonomy** is the science of naming things assigning them to particular groups
- **Classification** is the arrangement of those groups in a coherent order – which reflects their evolution and relatedness
- **Systematics** is the study of the diversity of organisms and the way they relate to each other

2. 16S rRNA gene sequencing

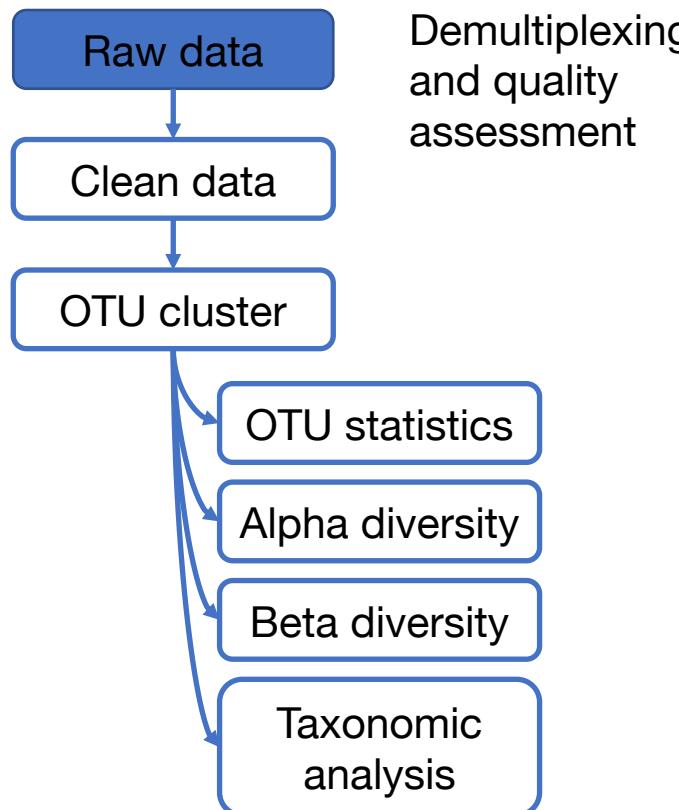


2. 16S rRNA gene sequencing



2. 16S rRNA gene sequencing

How are 16S sequence data analysed?



Analysis tools for targeted amplicon data

QIIME, *mothur* and *VAMPS* developed to allow the comparison of microbial communities

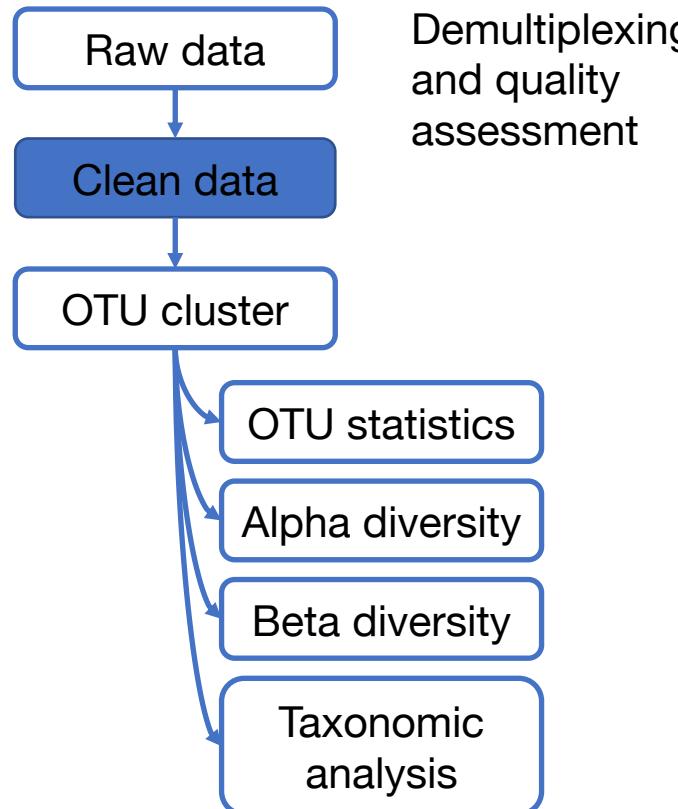
- **QIIME - Quantitative Insights Into Microbial Ecology**
<http://qiime.org>
- **Open-source bioinformatics pipeline**
 - Raw reads, identify rRNA, cluster to OTUs, taxon classification, diversity analysis, comparative statistics, various plots

Databases:

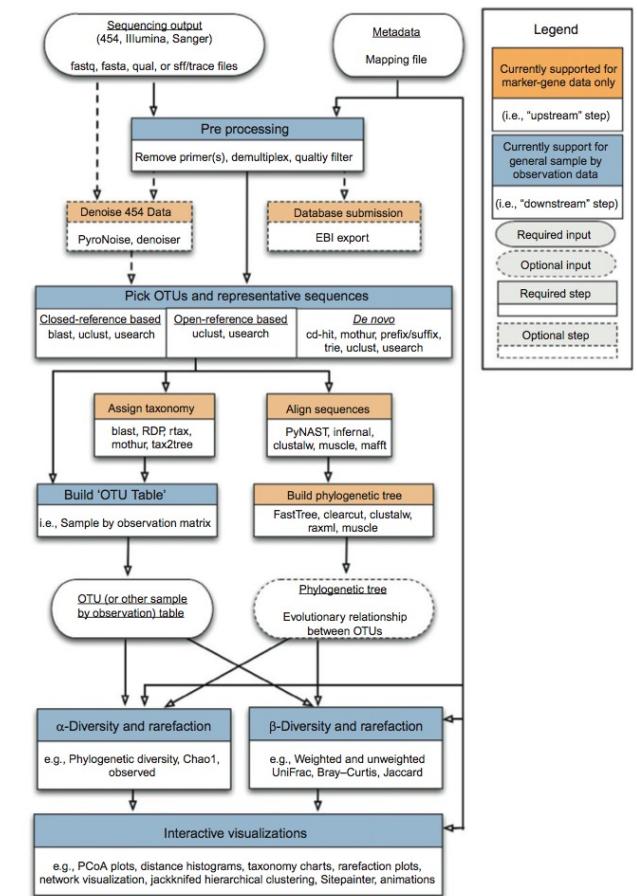
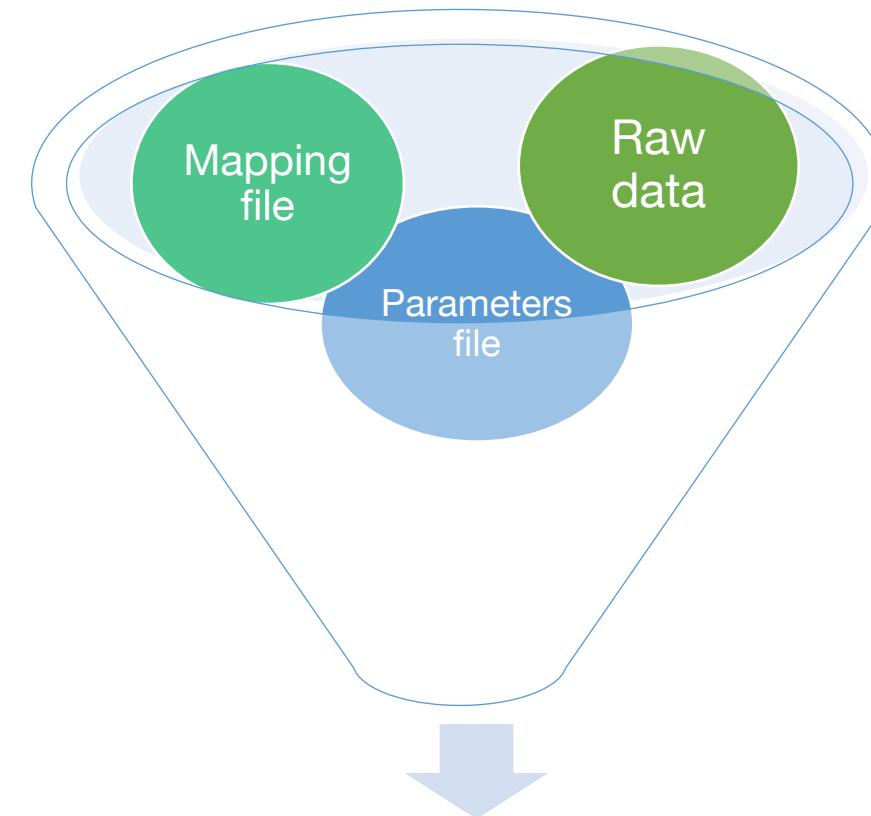
- Greengenes <http://greengenes.secondgenome.com/>
- SILVA <http://www.arb-silva.de/>

2. 16S rRNA gene sequencing

How are 16S sequence data analysed?

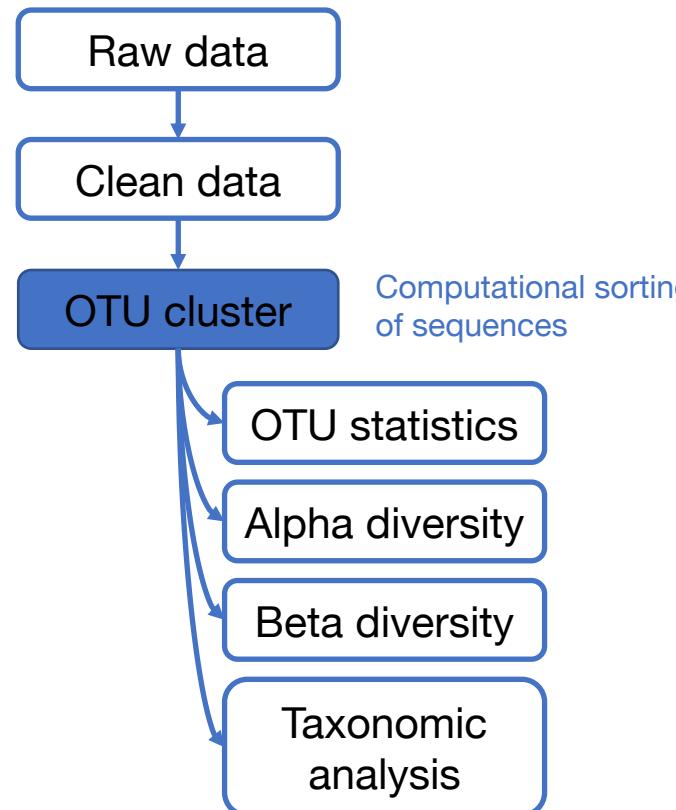


Analysis tools for targeted amplicon data workflow QIIME - Quantitative Insights Into Microbial Ecology



2. 16S rRNA gene sequencing

How are 16S sequence data analysed?



OTU: Operational Taxonomic Units

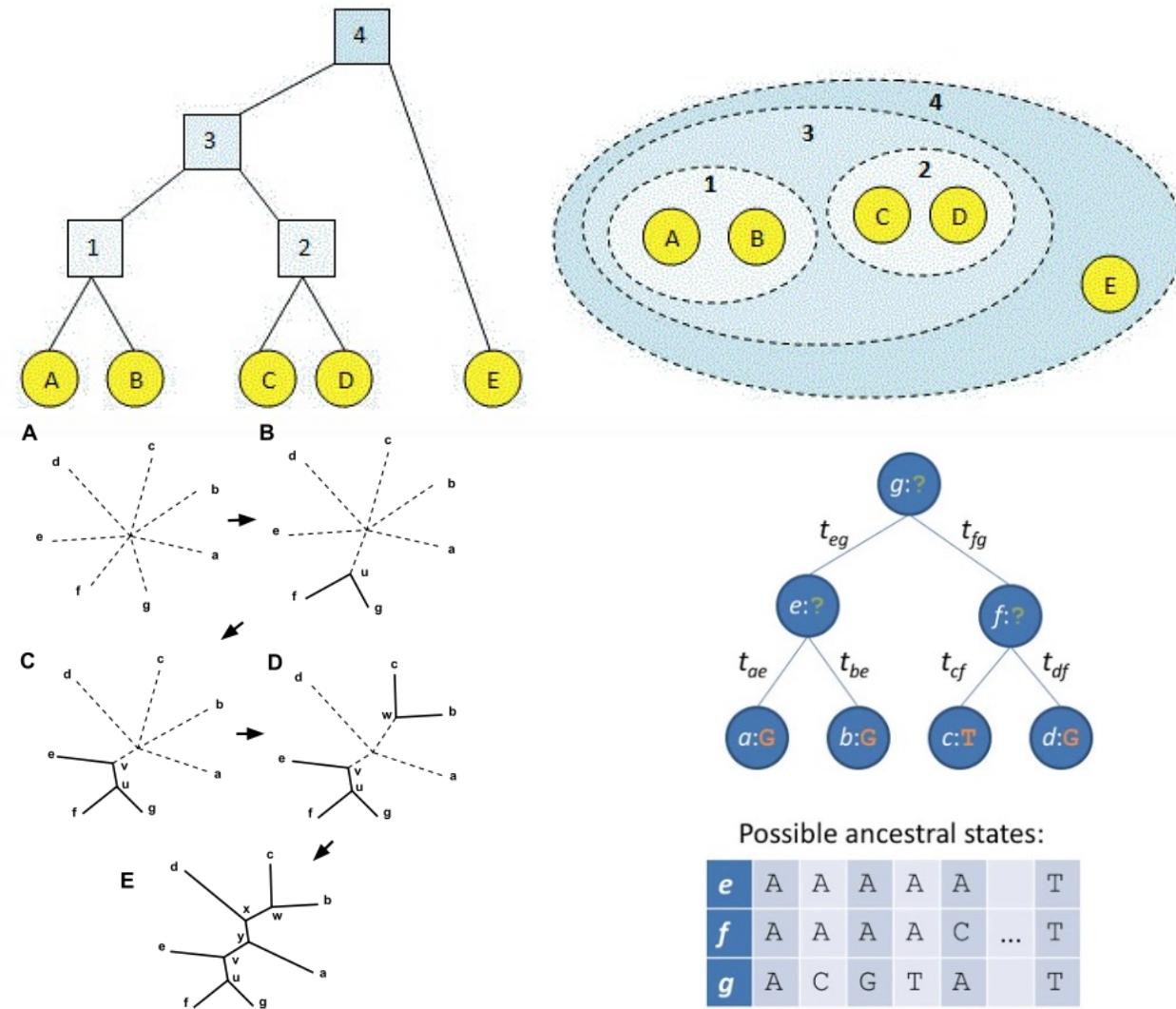
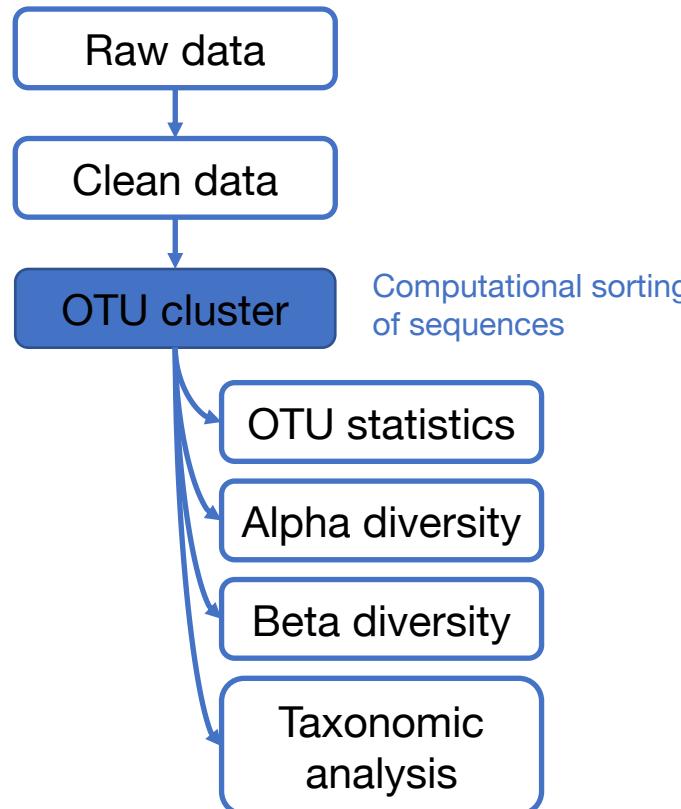
are used to represent groups of related organisms. OTUs at 3% sequence difference are used as a proxy for species-level diversity.

- The concept of an Operational Taxonomic Unit (OTU) was introduced by Sneath & Sokal in 1973.
- Quantitative strategy for classifying organisms into groups based on observed characters reflecting the evolutionary relationships between the organisms
- Table of observed traits which could be described by numerical values; e.g. 1=present, 0=absent

Ref. Sneath & Sokal: *Numerical Taxonomy*, W.H. Freeman, 1973

2. 16S rRNA gene sequencing

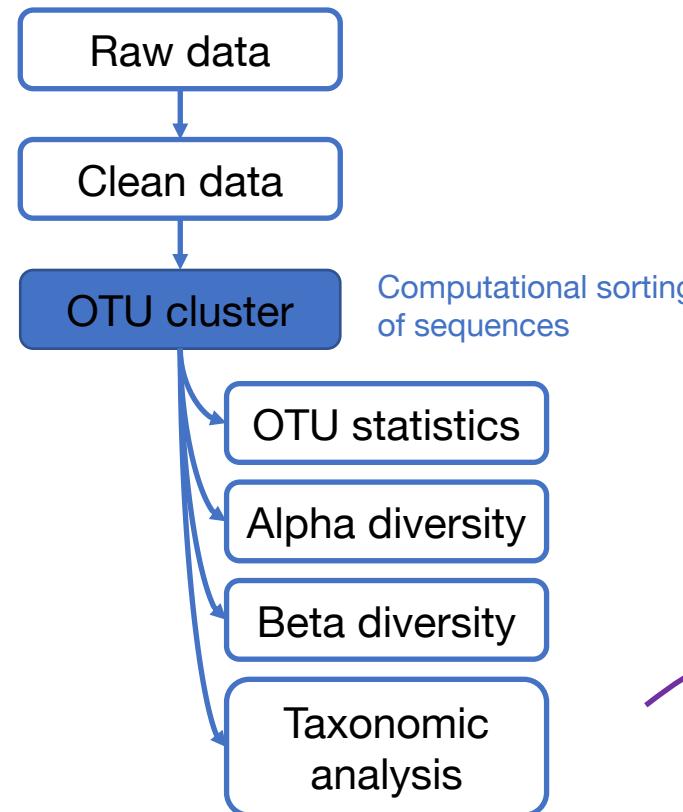
How are 16S sequence data analysed?



Ref. Sneath & Sokal: *Numerical Taxonomy*, W.H. Freeman, 1973

2. 16S rRNA gene sequencing

How are 16S sequence data analysed?



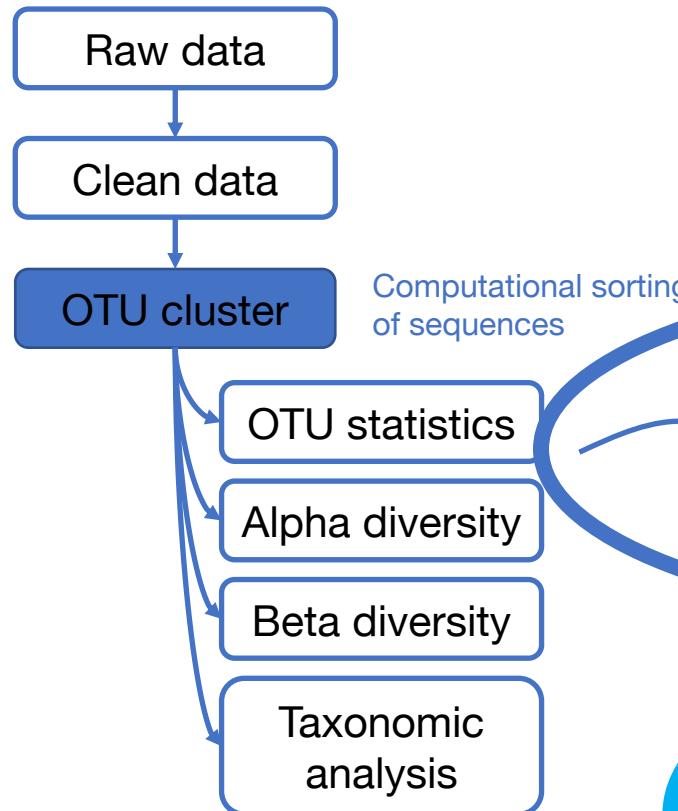
OTU: Operational Taxonomic Units

are used to represent groups of related organisms. OTUs at 3% sequence difference are used as a proxy for species-level diversity



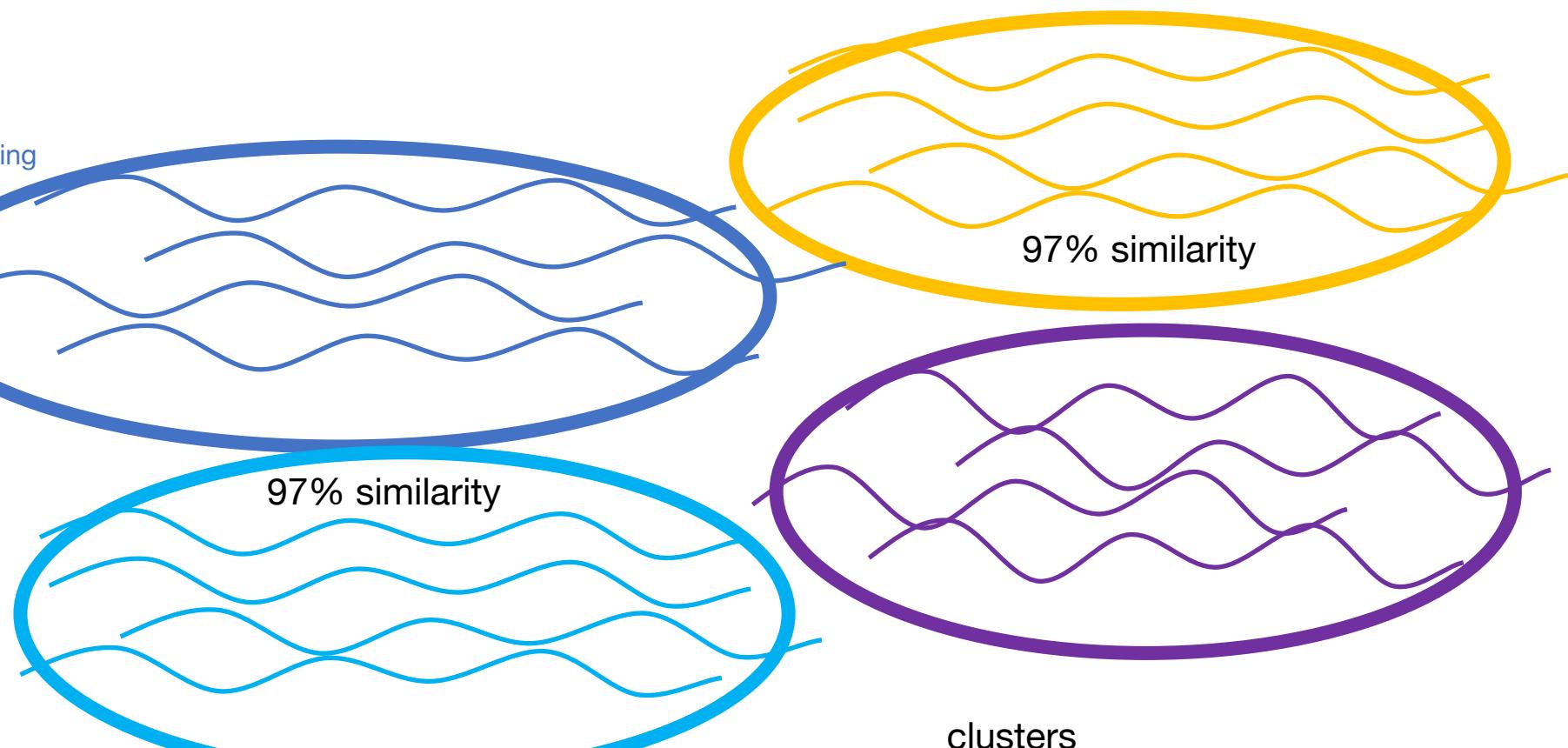
2. 16S rRNA gene sequencing

How are 16S sequence data analysed?



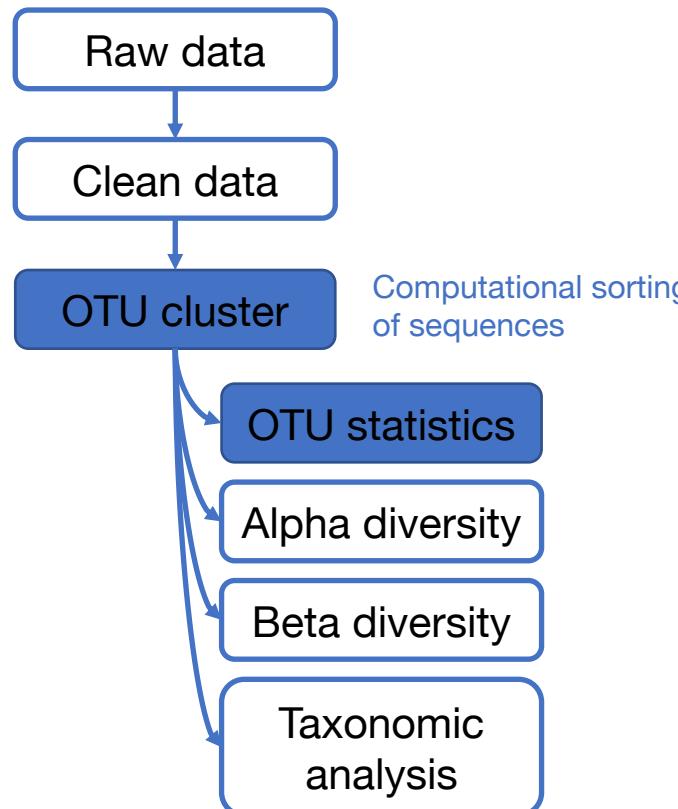
OTU: Operational Taxonomic Units

are used to represent groups of related organisms. OTUs at 3% sequence difference are used as a proxy for species-level diversity



2. 16S rRNA gene sequencing

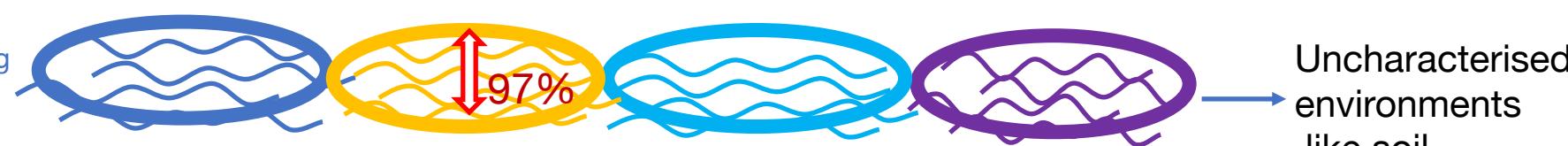
How are 16S sequence data analysed?



OTU: Operational Taxonomic Units

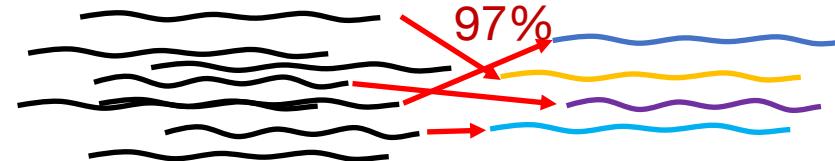
are used to represent groups of related organisms. OTUs at **3% sequence difference** are used as a proxy for species-level diversity

De Novo: reads are clustered based on the similarity to one another



Uncharacterised environments like soil

Closed reference: closest match in the database, discard failures

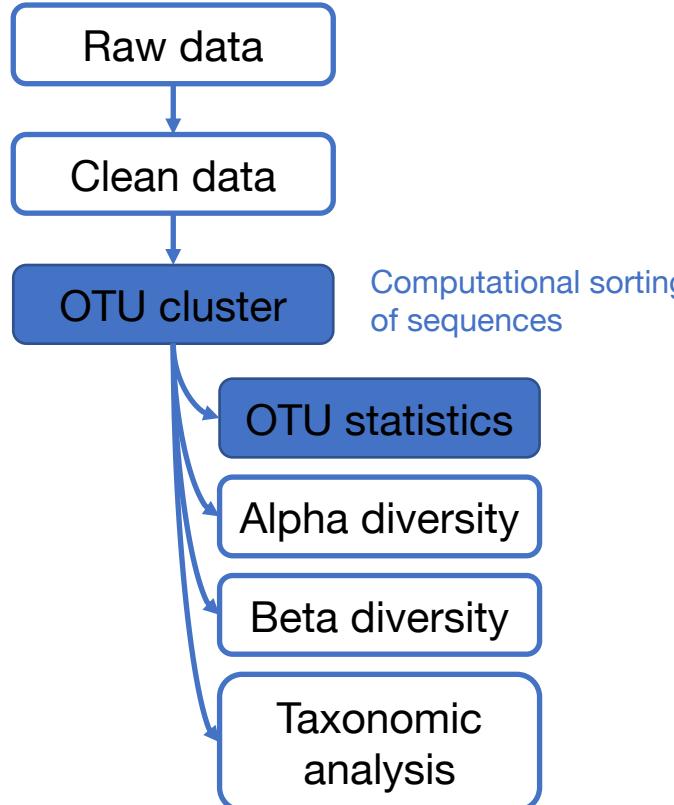


Human gut/skin/oral

clusters

2. 16S rRNA gene sequencing

OTU statistics

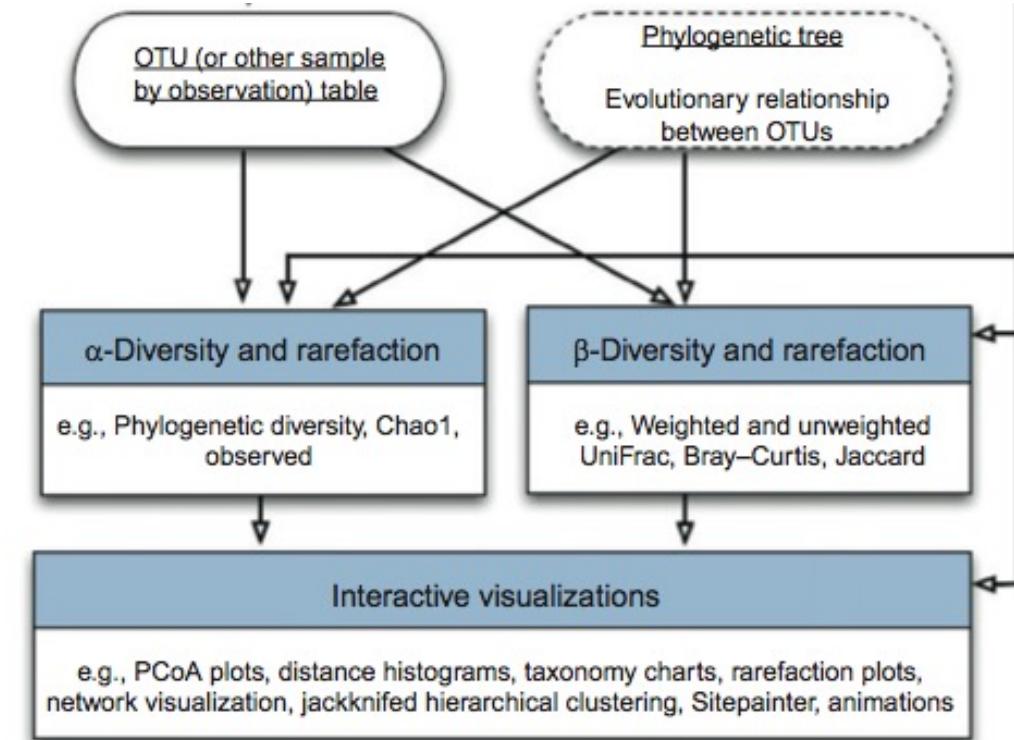
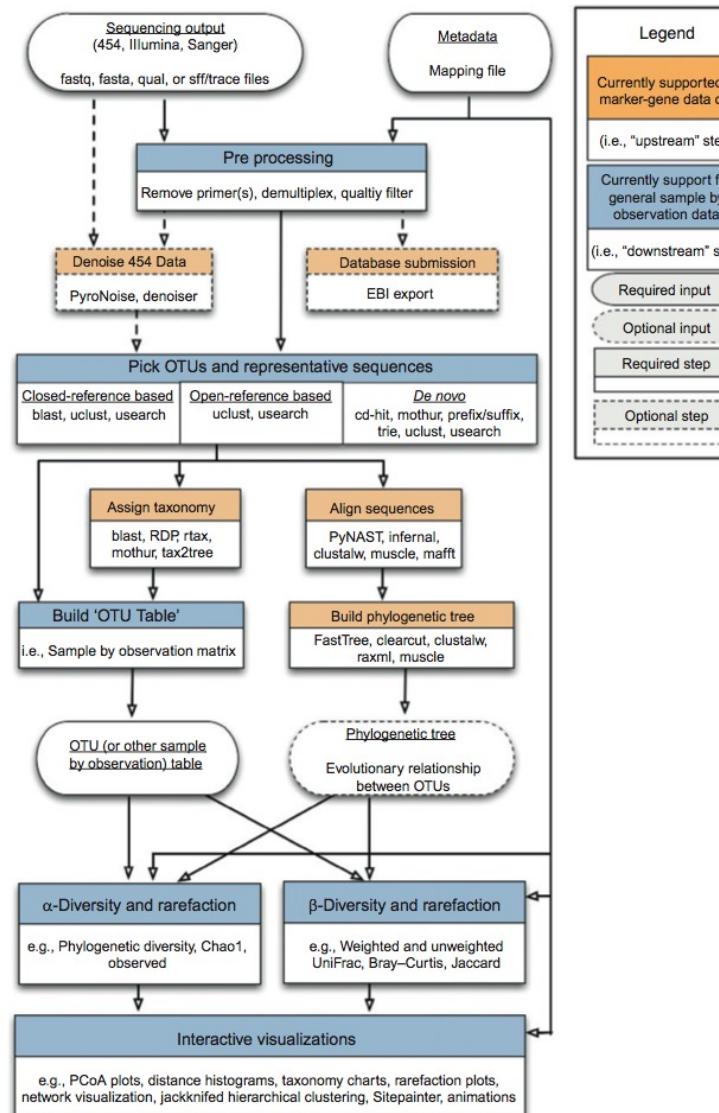
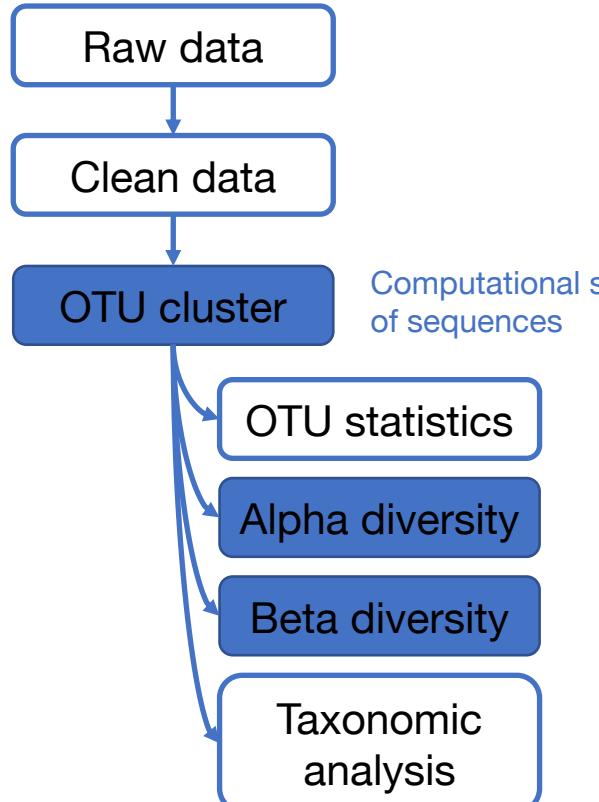


Assigning amplicon sequences to operational taxonomic units

| OTU ID | A_0_A | A_0_B | A_0_C | A_7_A | A_7_B | A_7_C | A_15_A | A_15_B | A_15_C |
|----------------------------------|-------|-------|-------|-------|-------|-------|--------|--------|--------|
| <i>Acinetobacter</i> | 10,98 | 0,75 | 0,63 | 16,56 | 0,06 | 0,38 | 7,09 | 0,38 | 0,00 |
| <i>Acinetobacter guillouiae</i> | 0,00 | 0,25 | 0,06 | 0,00 | 0,00 | 0,75 | 0,00 | 0,06 | 0,00 |
| <i>Acinetobacter johnsonii</i> | 35,67 | 34,33 | 35,07 | 4,39 | 0,06 | 65,75 | 7,09 | 80,80 | 7,65 |
| <i>Acinetobacter lwoffii</i> | 0,19 | 0,63 | 3,76 | 0,06 | 0,06 | 7,97 | 0,00 | 4,08 | 0,56 |
| <i>Aeromonadaceae</i> | 0,25 | 0,25 | 1,82 | 0,06 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Bacillus</i> | 0,00 | 0,44 | 0,00 | 0,13 | 0,00 | 0,00 | 0,00 | 0,00 | 0,06 |
| <i>Brochothrix</i> | 3,63 | 3,00 | 3,00 | 1,13 | 0,00 | 0,00 | 0,82 | 0,00 | 0,00 |
| <i>Macroccoccus caseolyticus</i> | 0,00 | 1,69 | 0,13 | 0,06 | 0,06 | 0,44 | 0,13 | 0,00 | 0,44 |
| <i>Caulobacteraceae</i> | 0,00 | 5,52 | 0,00 | 0,00 | 0,06 | 0,13 | 0,00 | 0,00 | 0,00 |
| <i>Chromohalobacter</i> | 0,00 | 0,00 | 0,00 | 0,06 | 0,25 | 0,00 | 0,00 | 0,00 | 0,25 |
| <i>Clostridium</i> | 0,00 | 3,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Halomonadaceae</i> | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,25 | 0,00 | 0,19 |
| <i>Halomonas</i> | 0,00 | 0,00 | 0,00 | 0,88 | 79,99 | 0,13 | 24,15 | 9,97 | 12,17 |
| <i>Kocuria</i> | 0,00 | 0,38 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,13 |
| <i>Lactobacillus</i> | 0,00 | 0,88 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Micrococcus</i> | 0,00 | 0,56 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Methylotenera mobilis</i> | 0,06 | 4,02 | 0,00 | 0,00 | 0,06 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Paracoccus</i> | 0,00 | 1,88 | 0,00 | 0,00 | 0,06 | 0,06 | 0,00 | 0,00 | 0,00 |
| <i>Propionibacterium acnes</i> | 0,00 | 0,88 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Pseudomonas</i> | 6,09 | 0,69 | 0,50 | 3,70 | 0,44 | 0,94 | 0,50 | 0,06 | 0,13 |
| <i>Pseudomonas fragi</i> | 20,14 | 25,00 | 26,00 | 68,13 | 2,13 | 0,31 | 48,93 | 0,31 | 0,00 |
| <i>Psychrobacter</i> | 1,19 | 0,94 | 0,06 | 2,63 | 2,20 | 0,56 | 5,08 | 0,13 | 0,00 |
| <i>Salinisphaera</i> | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Sphingomonas</i> | 0,00 | 0,88 | 0,00 | 0,00 | 0,06 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Staphylococcus</i> | 0,00 | 0,13 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| <i>Staphylococcus equorum</i> | 0,00 | 0,06 | 0,00 | 0,00 | 4,52 | 6,78 | 3,89 | 1,76 | 5,02 |
| <i>Staphylococcus sciuri</i> | 0,00 | 0,25 | 0,44 | 0,00 | 0,25 | 3,39 | 0,00 | 1,32 | 2,32 |
| <i>Staphylococcus succinus</i> | 0,00 | 0,00 | 0,00 | 0,00 | 0,44 | 0,06 | 0,00 | 0,19 | 0,06 |
| <i>Vibrio</i> | 0,00 | 0,19 | 0,00 | 0,00 | 0,00 | 10,79 | 0,06 | 0,06 | 68,63 |
| <i>Listeriaceae</i> | 0,06 | 2,53 | 0,00 | 0,00 | 0,38 | 0,13 | 0,00 | 0,00 | 0,19 |

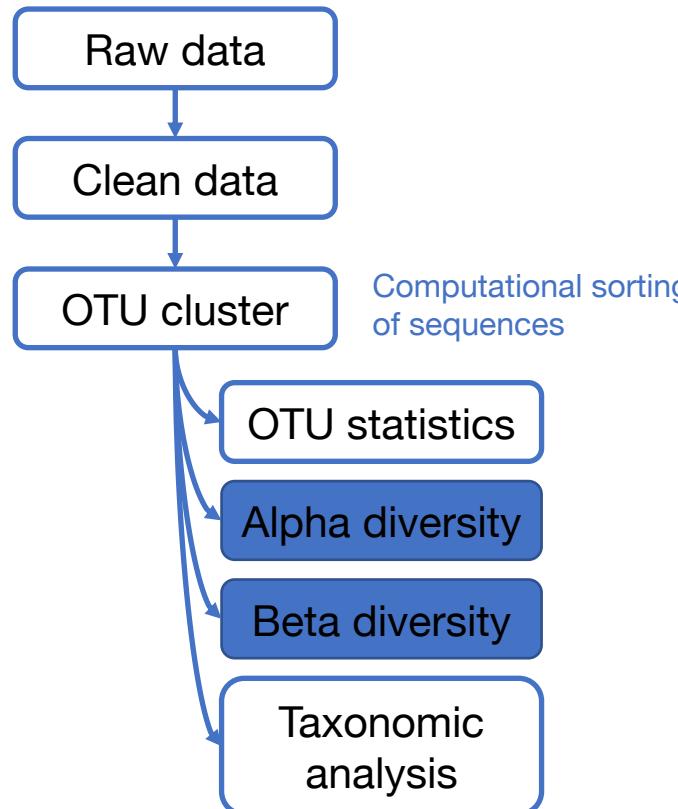
2. 16S rRNA gene sequencing

OTU statistics



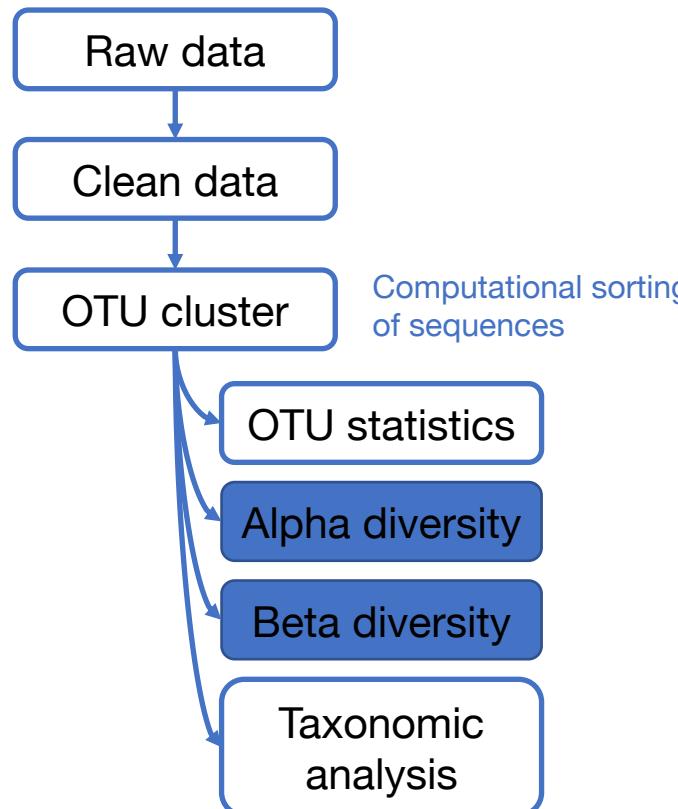
2. 16S rRNA gene sequencing

Diversity indexes



2. 16S rRNA gene sequencing

Diversity indexes

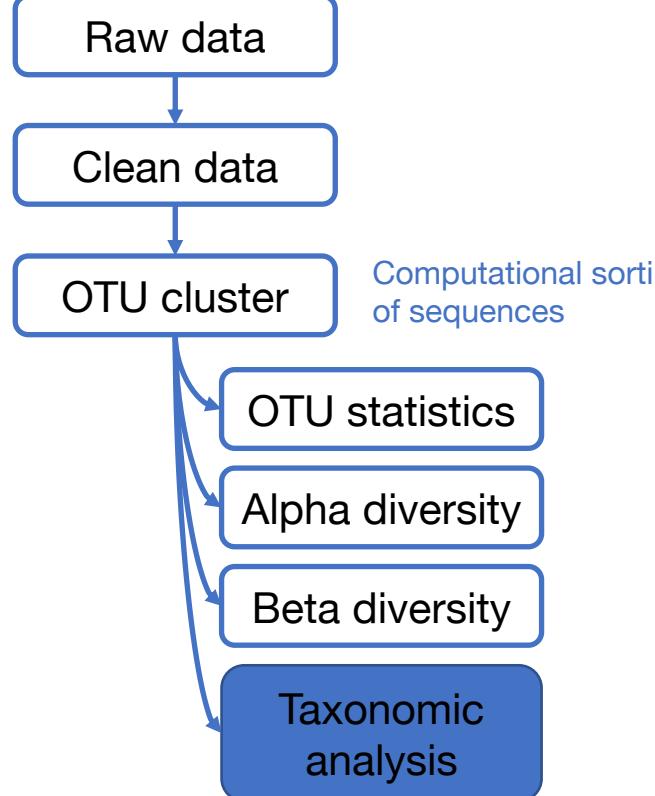


Beta diversity: difference between samples



2. 16S rRNA gene sequencing

Diversity indexes



| OTU | 0 days | | 7 days | | 15 days | | 30 days | | 60 days | | 90 days | |
|---------------------------------|---------------|------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|
| | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD |
| Plant A | | | | | | | | | | | | |
| <i>Acinetobacter</i> | 4.12 | 5.94 | 5.67 | 9.44 | 2.49 | 3.99 | 0.04 | 0.04 | 1.07 | 1.85 | 2.40 | 3.73 |
| <i>Acinetobacter guillouiae</i> | 0.10 | 0.13 | 0.25 | 0.43 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Acinetobacter johnsonii</i> | 35.02 | 0.67 | 23.40 | 36.74 | 31.85 | 42.40 | 3.01 | 2.80 | 0.61 | 0.89 | 8.01 | 12.15 |
| <i>Acinetobacter lwoffii</i> | 1.53 | 1.95 | 2.70 | 4.56 | 1.55 | 2.21 | 0.18 | 0.15 | 0.00 | 0.00 | 0.36 | 0.56 |
| <i>Aeromonadaceae</i> | 0.77 | 0.91 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Bacillus</i> | 0.15 | 0.25 | 0.04 | 0.07 | 0.02 | 0.04 | 0.00 | 0.00 | 0.19 | 0.33 | 0.04 | 0.04 |
| <i>Brochothrix</i> | 3.21 | 0.36 | 0.38 | 0.65 | 0.27 | 0.47 | 0.00 | 0.00 | 0.31 | 0.54 | 2.70 | 4.62 |
| <i>Caulobacteraceae</i> | 1.84 | 3.19 | 0.06 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.31 | 0.54 |
| <i>Chromohalobacter</i> | 0.00 | 0.00 | 0.10 | 0.13 | 0.08 | 0.14 | 1.75 | 0.92 | 0.38 | 0.39 | 17.29 | 16.46 |
| <i>Clostridium</i> | 1.07 | 1.85 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.07 |
| <i>Halomonadaceae</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.15 | 0.13 | 0.44 | 0.33 | 0.17 | 0.18 | 0.40 | 0.35 |
| <i>Halomonas</i> | 0.00 | 0.00 | 27.00 | 45.89 | 15.43 | 7.63 | 21.53 | 12.68 | 9.77 | 5.27 | 10.35 | 6.57 |
| <i>Kocuria</i> | 0.13 | 0.22 | 0.00 | 0.00 | 0.04 | 0.07 | 0.04 | 0.03 | 0.00 | 0.00 | 0.06 | 0.11 |
| <i>Lactobacillus</i> | 0.29 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.04 | 0.17 | 0.13 |
| <i>Listeriaceae</i> | 0.86 | 1.44 | 0.17 | 0.19 | 0.06 | 0.11 | 0.10 | 0.04 | 0.06 | 0.06 | 3.24 | 3.95 |
| <i>Macrococcus caseolyticus</i> | 0.61 | 0.94 | 0.19 | 0.22 | 0.19 | 0.23 | 0.00 | 0.00 | 0.00 | 0.00 | 0.44 | 0.76 |
| <i>Methylotenera mobilis</i> | 1.36 | 2.30 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.48 | 0.83 |
| <i>Micrococcus</i> | 0.19 | 0.33 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.04 |
| <i>Paracoccus</i> | 0.63 | 1.09 | 0.04 | 0.04 | 0.00 | 0.00 | 0.03 | 0.03 | 0.02 | 0.04 | 0.44 | 0.60 |
| <i>Propionibacterium acnes</i> | 0.29 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Pseudomonas</i> | 2.43 | 3.17 | 1.69 | 1.76 | 0.23 | 0.24 | 0.05 | 0.04 | 3.01 | 5.22 | 0.13 | 0.06 |
| <i>Pseudomonas fragi</i> | 23.71 | 3.14 | 23.53 | 38.64 | 16.42 | 28.16 | 0.05 | 0.05 | 6.13 | 10.61 | 12.61 | 21.35 |
| <i>Psychrobacter</i> | 0.73 | 0.59 | 1.80 | 1.09 | 1.74 | 2.90 | 0.18 | 0.11 | 2.66 | 4.49 | 9.60 | 11.10 |
| <i>Salinisphaera</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.36 | 2.35 |
| <i>Sphingomonas</i> | 0.29 | 0.51 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.13 | 0.22 |
| <i>Staphylococcus</i> | 0.04 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.40 | 1.96 |
| <i>Staphylococcus equorum</i> | 0.02 | 0.04 | 3.76 | 3.45 | 3.55 | 1.66 | 1.78 | 1.97 | 0.77 | 0.63 | 12.17 | 7.21 |
| <i>Staphylococcus sciuri</i> | 0.23 | 0.22 | 1.21 | 1.89 | 1.21 | 1.16 | 0.71 | 0.62 | 0.02 | 0.04 | 5.29 | 9.06 |
| <i>Staphylococcus succinus</i> | 0.00 | 0.00 | 0.17 | 0.24 | 0.08 | 0.10 | 0.08 | 0.07 | 0.02 | 0.04 | 0.52 | 0.44 |
| <i>Vibrio</i> | 0.06 | 0.11 | 3.60 | 6.23 | 22.92 | 39.59 | 64.50 | 6.16 | 65.73 | 16.10 | 3.79 | 6.34 |

2. 16S rRNA gene sequencing

Protected designation of origin (PDO) product: Lardo - *Valle d'Aosta Lard d'Arnad*
is a type of salumi made by curing strips of fatback with rosemary and other herbs and spices



| OTU | 0 days | | 7 days | | 15 days | | 30 days | | 60 days | | 90 days | |
|-----------------------------------|---------------|------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|
| | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD | Abundance (%) | SD |
| Plant A | | | | | | | | | | | | |
| <i>Acinetobacter</i> | 4.12 | 5.94 | 5.67 | 9.44 | 2.49 | 3.99 | 0.04 | 0.04 | 1.07 | 1.85 | 2.40 | 3.73 |
| <i>Acinetobacter guillouiae</i> | 0.10 | 0.13 | 0.25 | 0.43 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Acinetobacter johnsonii</i> | 35.02 | 0.67 | 23.40 | 36.74 | 31.85 | 42.40 | 3.01 | 2.80 | 0.61 | 0.89 | 8.01 | 12.15 |
| <i>Acinetobacter iwoffii</i> | 1.53 | 1.95 | 2.70 | 4.54 | 0.00 | 2.21 | 0.18 | 0.00 | 0.00 | 0.00 | 0.36 | 0.56 |
| <i>Arenimonadaceae</i> | 0.77 | 0.09 | 0.12 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Bacillus</i> | 0.15 | 0.25 | 0.04 | 0.07 | 0.02 | 0.04 | 0.00 | 0.00 | 0.19 | 0.33 | 0.04 | 0.04 |
| <i>Brochothrix</i> | 3.21 | 0.36 | 0.38 | 0.65 | 0.27 | 0.47 | 0.00 | 0.00 | 0.31 | 0.54 | 2.70 | 4.62 |
| <i>Caulobacteraeae</i> | 1.84 | 3.19 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.31 | 0.54 |
| <i>Chromohalobacter</i> | 0.00 | 0.00 | 0.10 | 0.13 | 0.08 | 0.14 | 1.75 | 0.92 | 0.38 | 0.39 | 17.29 | 16.46 |
| <i>Dermatophilus</i> | 0.07 | 1.85 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 |
| <i>Holomonsellaceae</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.15 | 0.13 | 0.44 | 0.33 | 0.17 | 0.18 | 0.40 | 0.35 |
| <i>Holomonas</i> | 0.00 | 0.00 | 27.00 | 45.89 | 15.43 | 7.63 | 21.53 | 12.68 | 9.77 | 5.27 | 10.35 | 6.57 |
| <i>Kocuria</i> | 0.13 | 0.22 | 0.00 | 0.00 | 0.04 | 0.07 | 0.04 | 0.00 | 0.00 | 0.00 | 0.06 | 0.11 |
| <i>Lactobacillus</i> | 0.29 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.04 | 0.17 | 0.13 |
| <i>Listeriacae</i> | 0.86 | 1.44 | 0.17 | 0.19 | 0.06 | 0.11 | 0.10 | 0.04 | 0.06 | 0.06 | 3.24 | 3.95 |
| <i>Methylophilus caseolyticus</i> | 0.01 | 0.04 | 0.00 | 0.21 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.44 | 0.76 |
| <i>Methylstearina mobilis</i> | 0.36 | 2.39 | 0.02 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.49 | 0.83 |
| <i>Micrococcus</i> | 0.19 | 0.33 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.04 |
| <i>Parococcus</i> | 0.63 | 1.09 | 0.04 | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.02 | 0.04 | 0.44 | 0.60 |
| <i>Propionibacterium acnes</i> | 0.29 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| <i>Pseudomonas</i> | 2.43 | 3.17 | 1.69 | 1.76 | 0.23 | 0.24 | 0.05 | 0.04 | 3.01 | 5.22 | 0.13 | 0.06 |
| <i>Pseudomonas fragi</i> | 0.71 | 2.14 | 0.43 | 38.64 | 0.26 | 20.45 | 0.05 | 0.05 | 1.3 | 10.21 | 21.35 | 21.35 |
| <i>Pycnophytrebacter</i> | 0.73 | 0.59 | 1.80 | 1.00 | 1.74 | 2.90 | 0.18 | 0.11 | 2.66 | 4.49 | 9.60 | 11.10 |
| <i>Salinosporea</i> | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.36 | 2.35 |
| <i>Sphingomonas</i> | 0.29 | 0.51 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.13 | 0.22 |
| <i>Staphylococcus</i> | 0.04 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.40 | 1.96 |
| <i>Staphylococcus equorum</i> | 0.02 | 0.04 | 3.76 | 3.45 | 3.55 | 1.66 | 1.78 | 1.97 | 0.77 | 0.63 | 12.17 | 7.21 |
| <i>Staphylococcus sciuri</i> | 0.22 | 0.22 | 0.01 | 1.80 | 1.21 | 1.00 | 0.70 | 0.02 | 0.00 | 0.00 | 5.00 | 5.06 |
| <i>Staphylococcus succinus</i> | 0.00 | 0.00 | 0.17 | 0.24 | 0.08 | 0.10 | 0.08 | 0.07 | 0.02 | 0.04 | 0.52 | 0.44 |
| <i>Vibrio</i> | 0.06 | 0.11 | 3.60 | 6.23 | 22.92 | 39.59 | 64.50 | 6.16 | 65.73 | 16.10 | 3.79 | 6.34 |

Material and Methods:

Three plants:

1. low maturation temperature (plant A [10% NaCl, 2°C]) x3
2. using a low NaCl concentration (plant B [2.5% NaCl, 4°C]) x3
3. artisanal process (plant C [30% NaCl, 8°C]) x3

Experimental design:

Lard samples were obtained at time 0 and after 7, 15, 30, 60, and 90 days of maturation.
16S rRNA gene sequencing (V3-V4 regions) and microbiology parameters

Ferrucino et al., 2017 doi:10.1128/AEM.00983-17

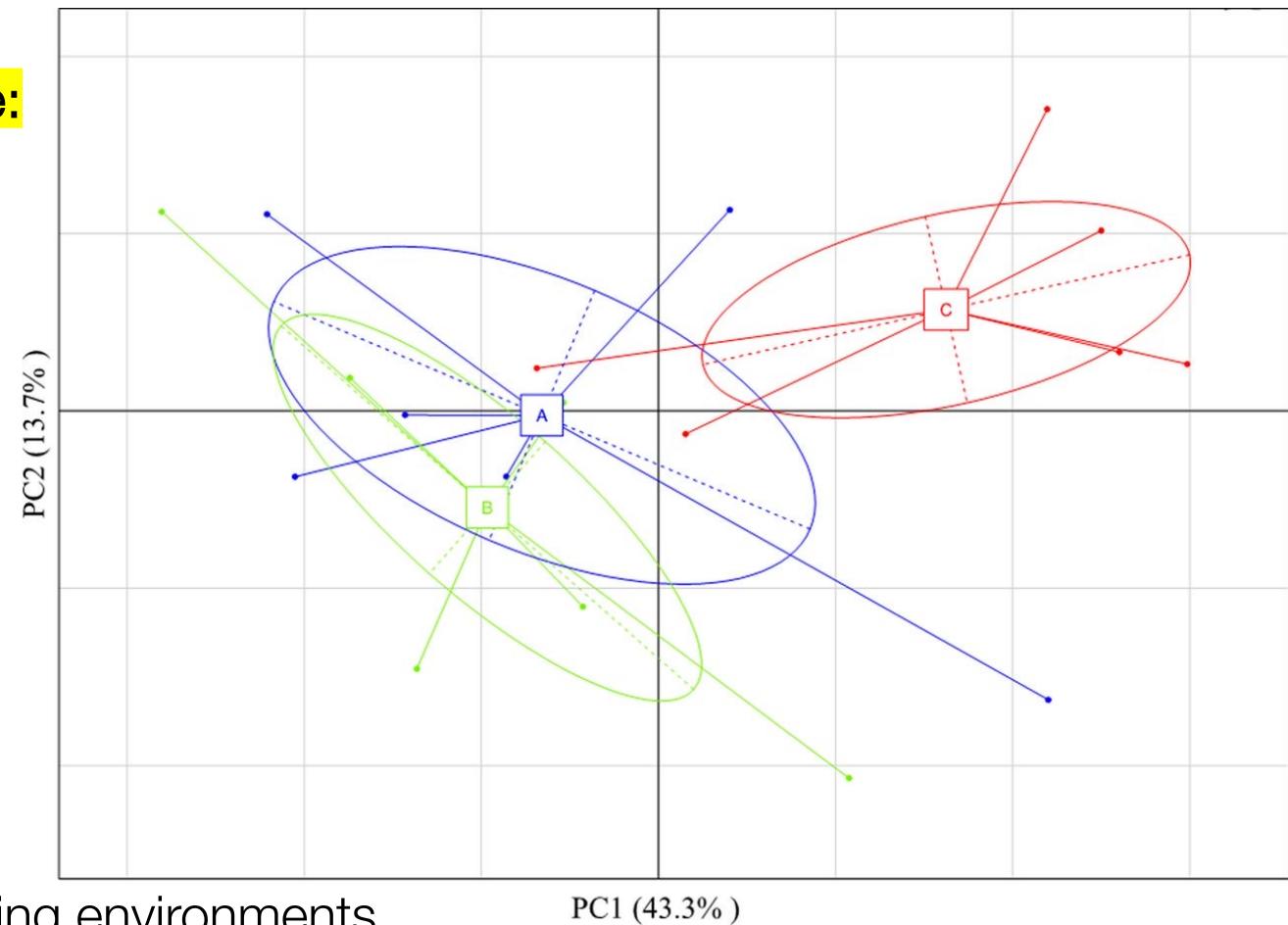
2. 16S rRNA gene sequencing



An example: a protected designation of origin (PDO) product

Main taxa identified by sequencing were:

Acinetobacter johnsonii,
Psychrobacter,
Staphylococcus equorum,
Staphylococcus sciuri,
Pseudomonas fragi,
Brochothrix,
Halomonas,
Vibrio



Relative abundances from the plants

A-B . Undesired bacteria in food-processing environments

Ferrocino et al., 2017 doi:10.1128/AEM.00983-17

2. 16S rRNA gene sequencing



An example: a protected designation of origin (PDO) product

Main taxa identified by sequencing were:

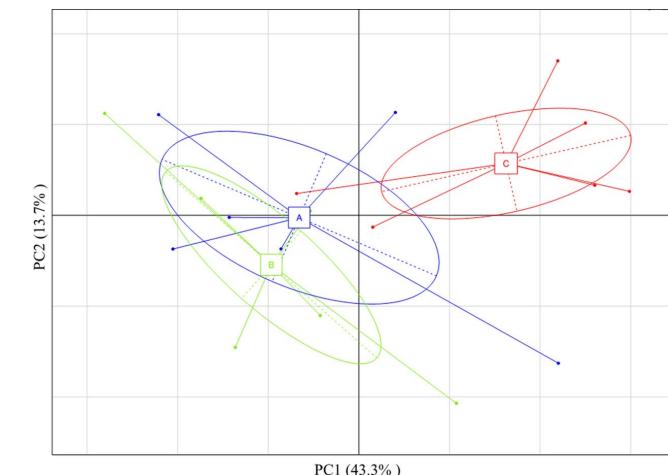
- ● *Acinetobacter johnsonii*
- *Psychrobacter,*
Staphylococcus equorum,
- *Staphylococcus sciuri,*
- ● *Pseudomonas fragi*
- ● *Brochothrix,*
- ● *Halomonas*
- *Vibrio*

-> spoilage agent: lipolytic activity

-> spoilage agent: lipolytic activity

-> spoilage agent: lipolytic potential (salt-resistant)

Acinetobacter johnsonii was also found in C samples but was not detected at the end of the ripening [concentrations of sodium chloride]



2. 16S rRNA gene sequencing



An example: a protected designation of origin (PDO) product

The use of 16S rRNA gene sequencing for technological improvement and provide products with reduced content of salt

- Changes in the food production process can drastically affect the microbial community structure
- Impact on the final characteristics of the products

Importance:

- Reduction in the salt concentration in the brines to address a consumer demand for less salty products can negatively impact the quality of the final product due to the higher abundance of spoilage bacteria.
- Importance of the use of traditional process to produce PDO from a spoilage perspective.

2. 16S rRNA gene sequencing



An example: a protected designation of origin (PDO) product

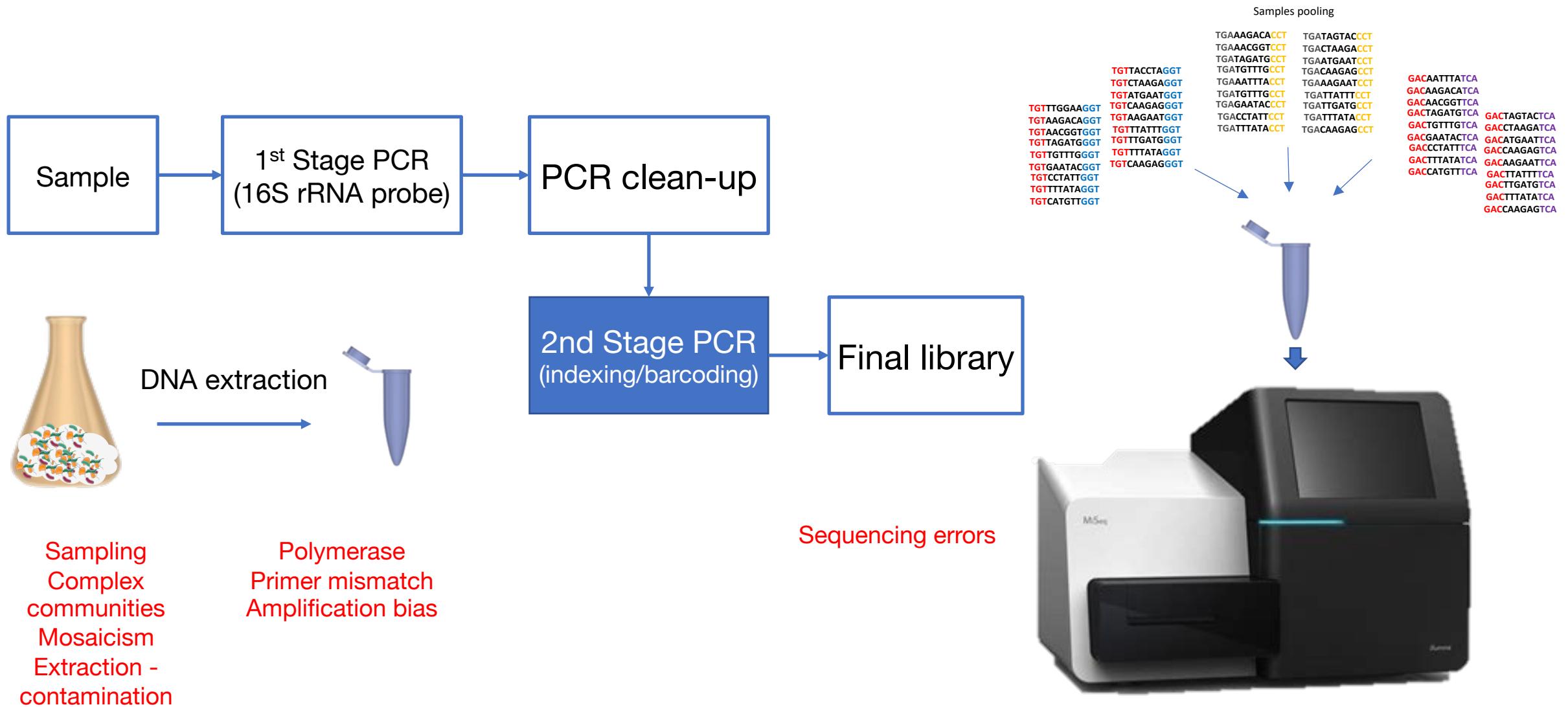
The use of 16S rRNA gene sequencing for technological improvement and provide products with reduced content of salt

- Changes in the food production process can drastically affect the microbial community structure
- Impact on the final characteristics of the products

Importance:

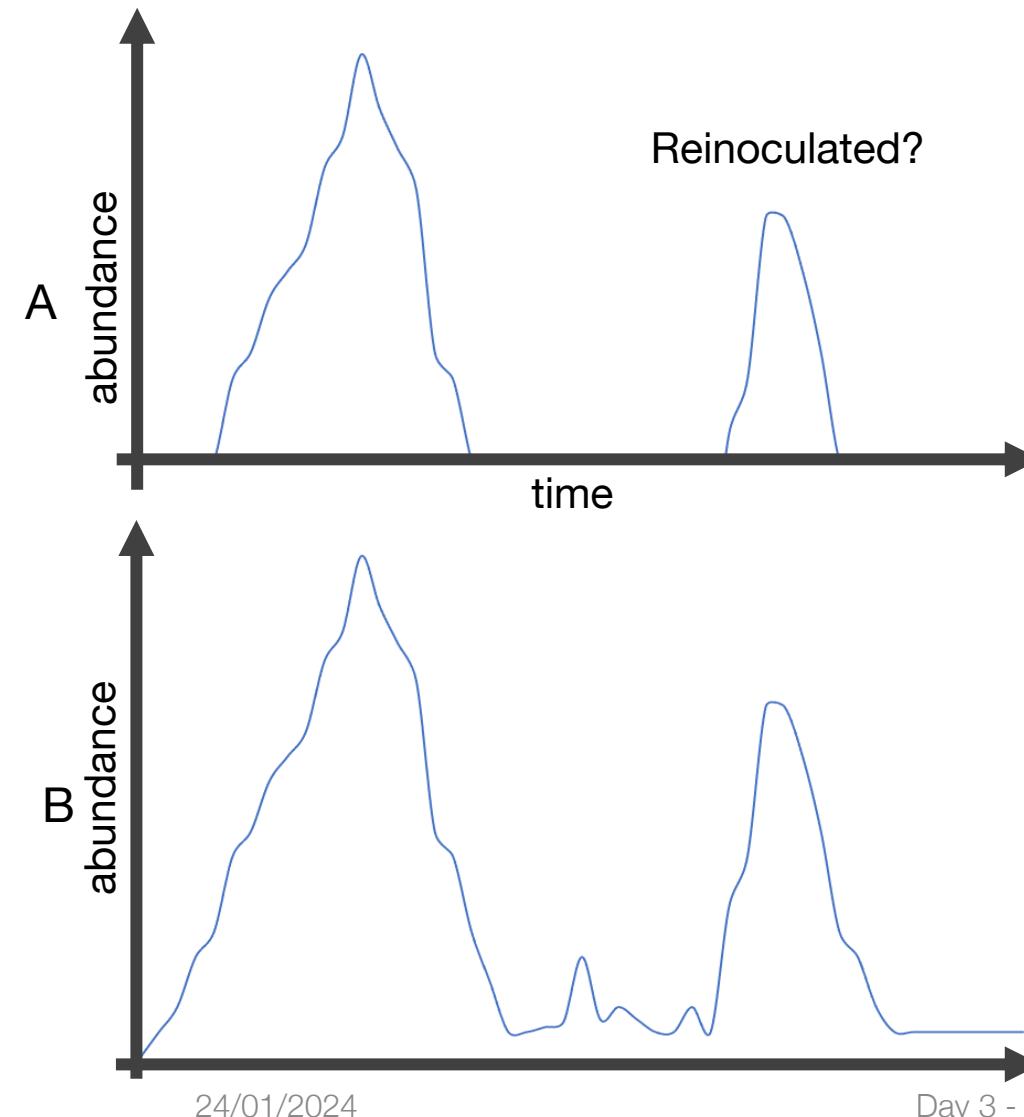
- Reduction in the salt concentration in the brines to address a consumer demand for less salty products can negatively impact the quality of the final product due to the higher abundance of spoilage bacteria.
- Importance of the use of traditional process to produce PDO from a spoilage perspective.

2. 16S rRNA gene sequencing



2. 16S rRNA gene sequencing

Deep Sequencing and complex populations

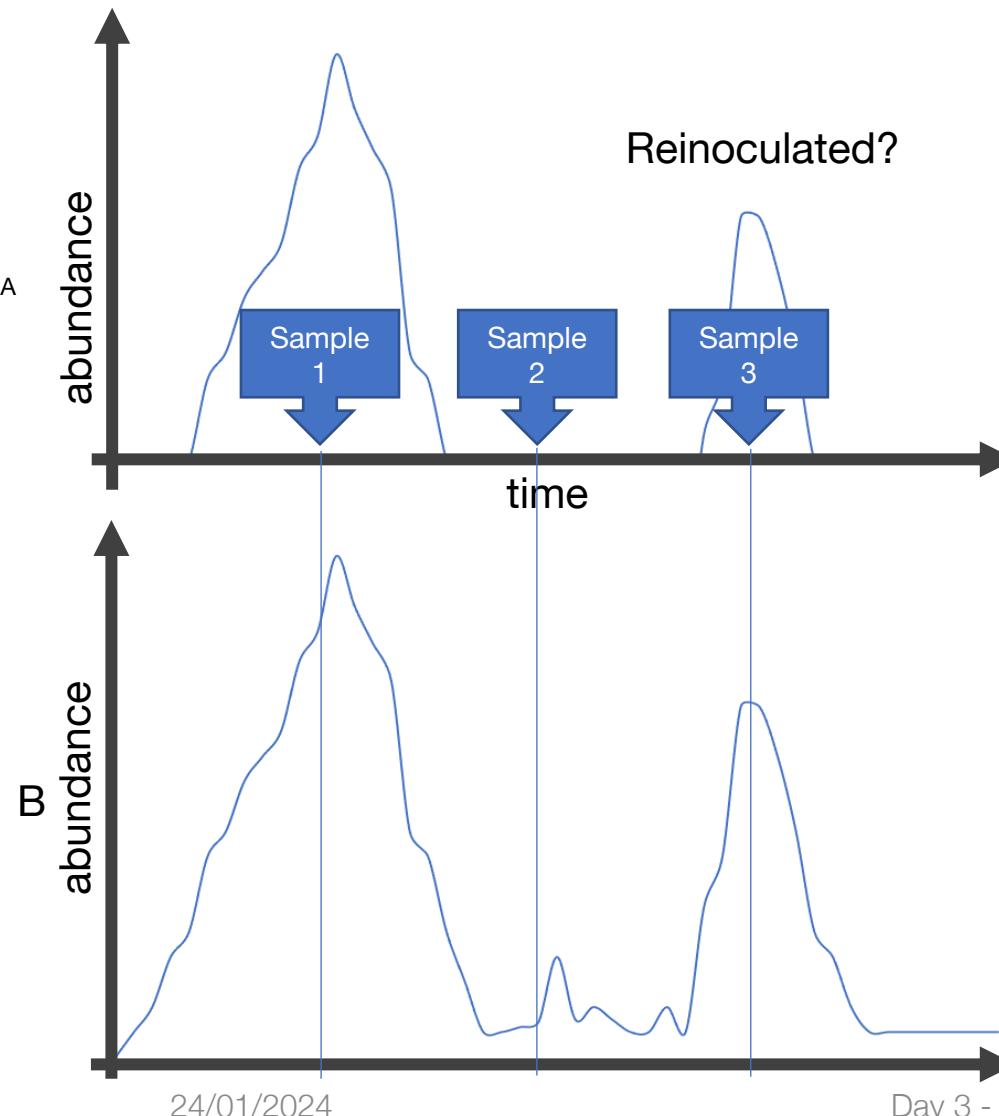


One of the most important advantages of NGS is the **amount of sequence information it can produce.**

- **Deep sequencing** refers to the sequencing of a genomic region multiple times—typically hundreds or even thousands of times.
- This makes it possible to detect organisms that exist in very low abundance within complex populations.
- Panels A and B: same microbial population sampled at two depths.
- Panel A, it appears that the microbes were reintroduced from an external source.
- Deep sequencing in panel B reveals that the microbes were present at all time-points, but dropped below the detection level used in panel A.

2. 16S rRNA gene sequencing

Deep Sequencing and complex populations

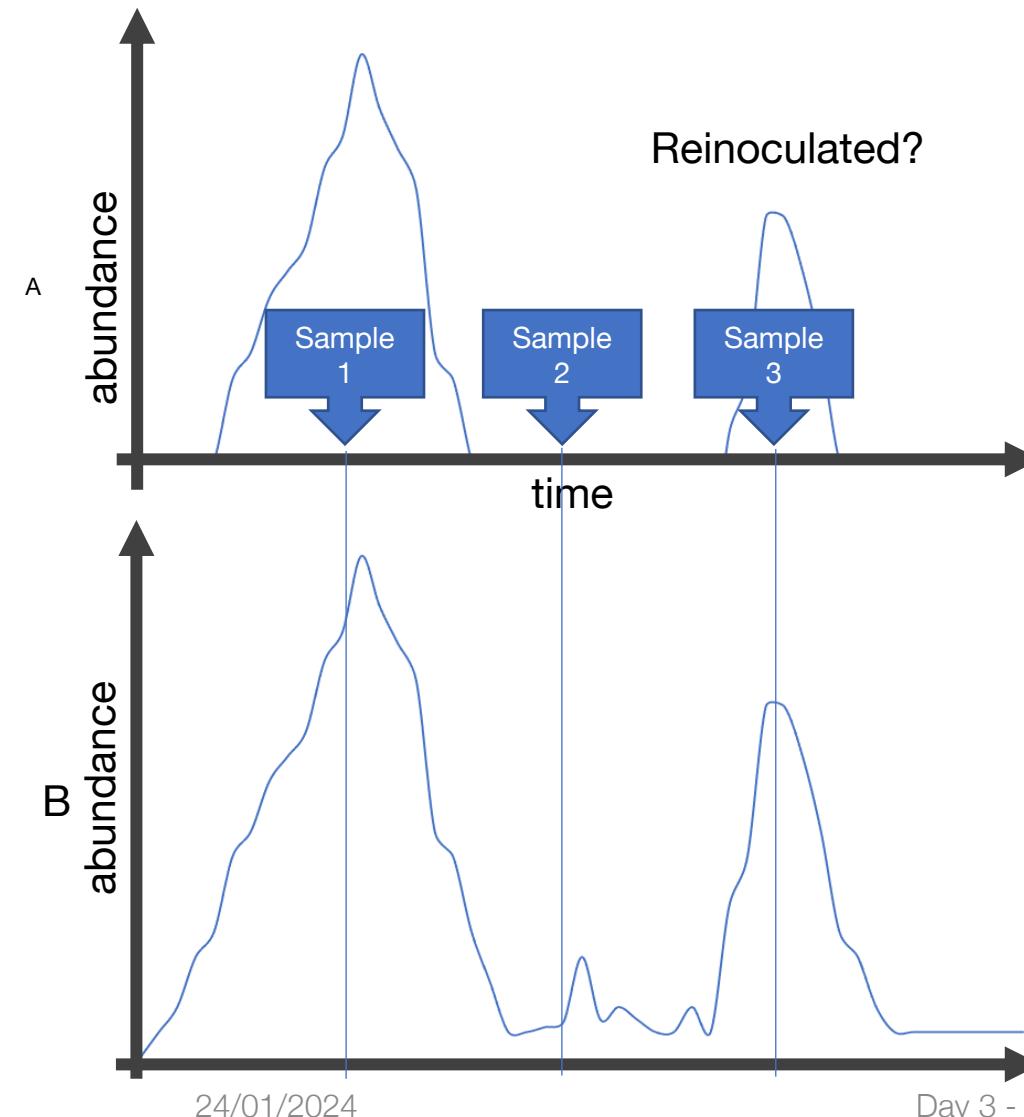


One of the most important advantages of NGS is the **amount of sequence information it can produce.**

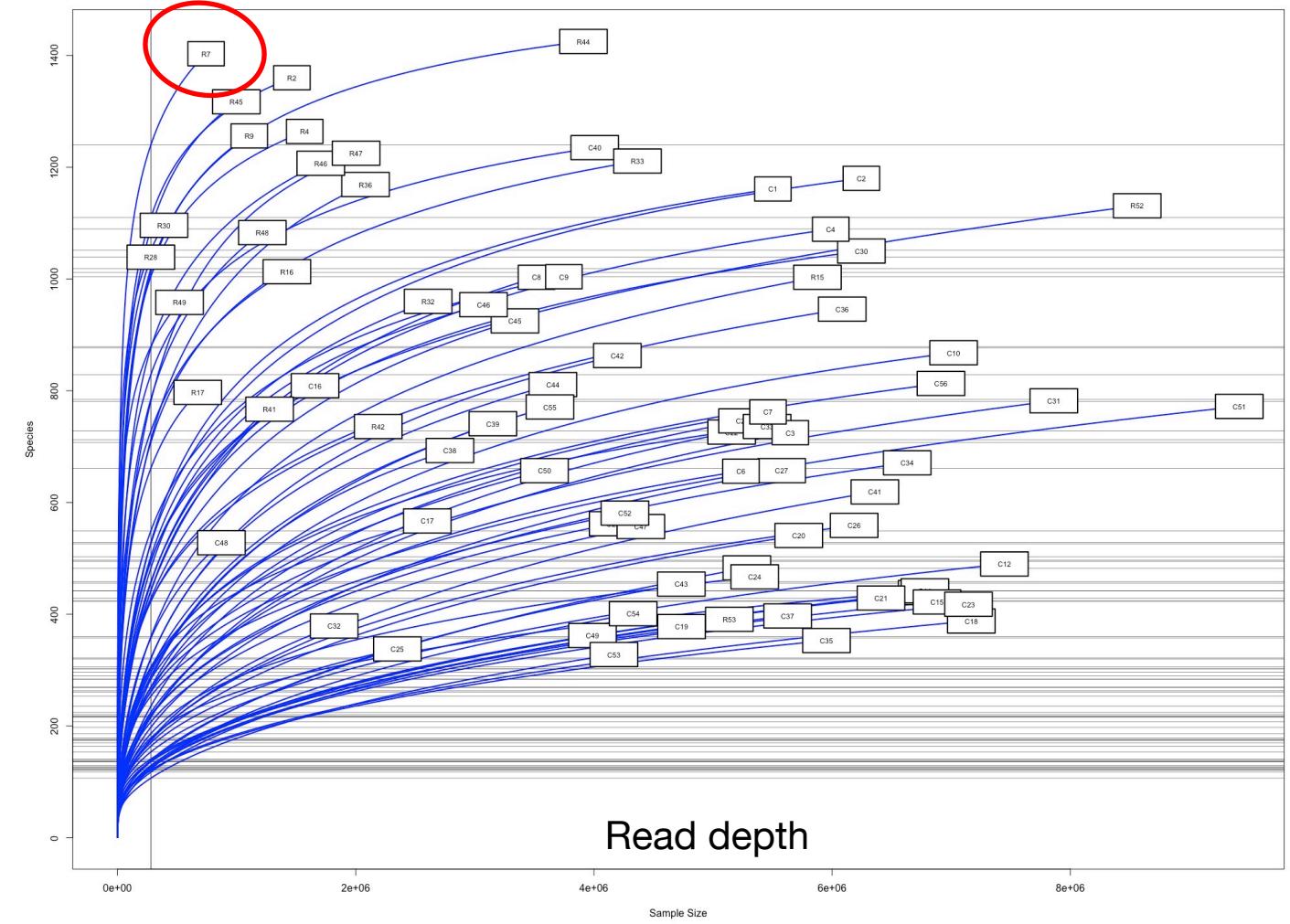
- **Deep sequencing** refers to the sequencing of a genomic region multiple times—typically hundreds or even thousands of times.
- This makes it possible to detect organisms that exist in very low abundance within complex populations.
- Panels A and B: same microbial population sampled at two depths.
- Panel A, it appears that the microbes were reintroduced from an external source.
- Deep sequencing in panel B reveals that the microbes were present at all time-points, but dropped below the detection level used in panel A.

2. 16S rRNA gene sequencing

Deep Sequencing and complex populations



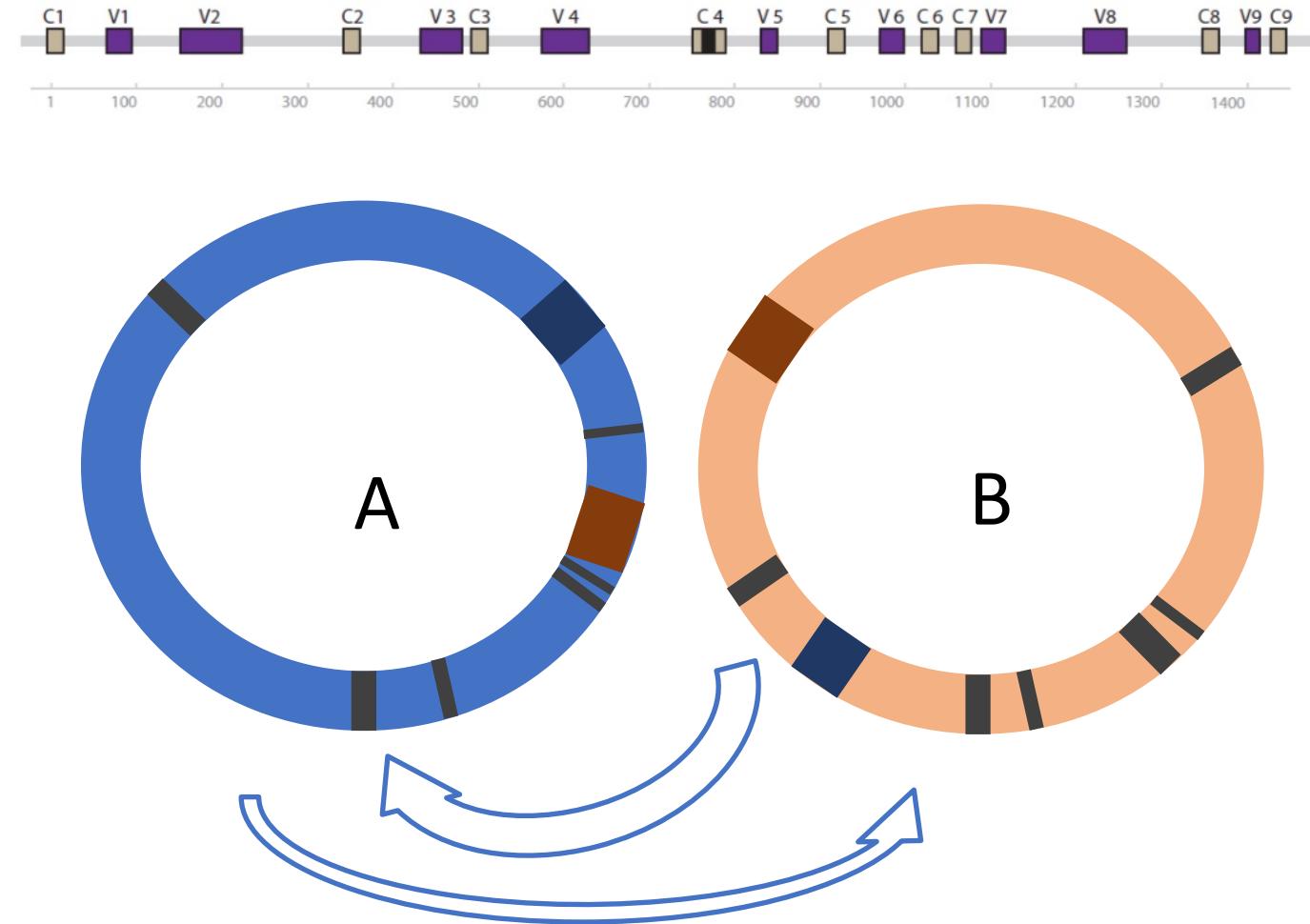
Rarefaction curve - to estimate the species richness as a function of sampling (sequencing depth).



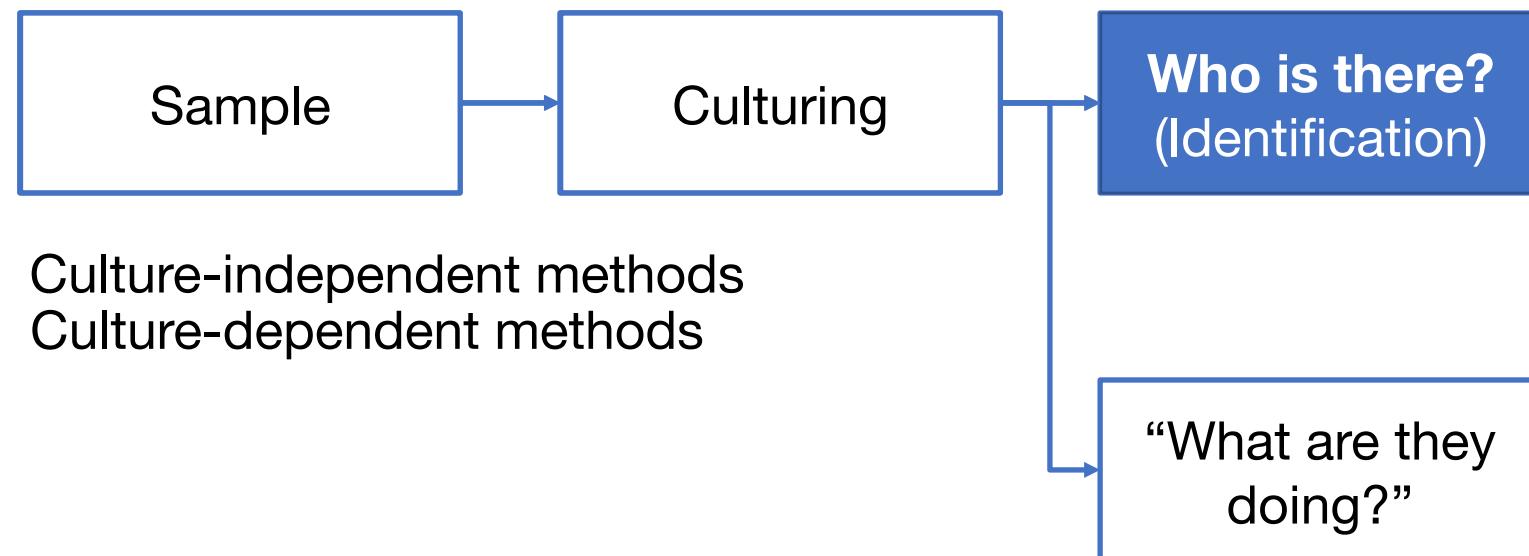
2. 16S rRNA gene sequencing

Deep Sequencing and complex populations

- The variable regions are different sizes and can change at different rates; for example, the variable region V6 appear to systematically overestimate species richness.
- Horizontal Gene Transfer: bacteria can tolerate transfer of complete 16S genes resulting in misidentification



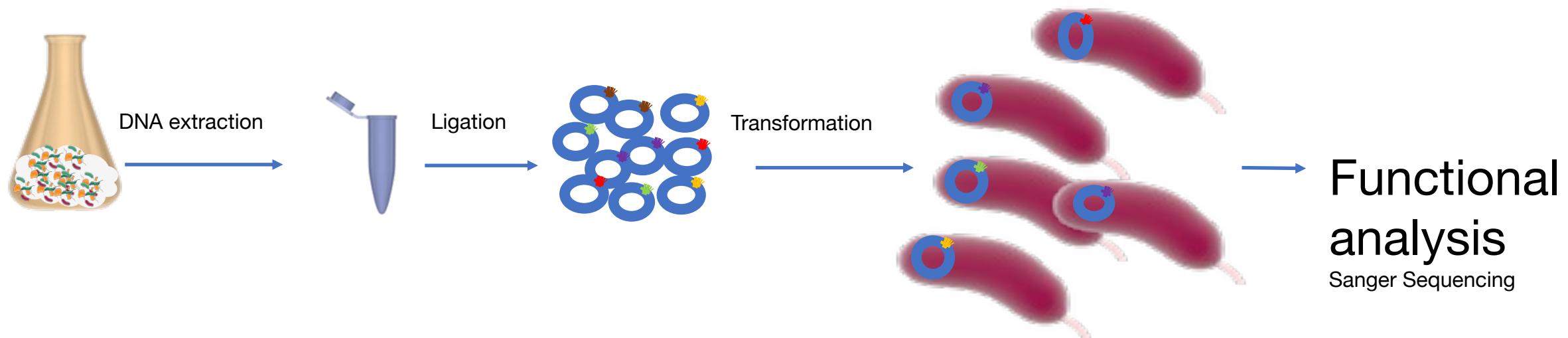
3. Shotgun metagenomics sequencing



3. Shotgun metagenomics sequencing

Metagenomics is the technique of retrieving microbial genome directly from the samples

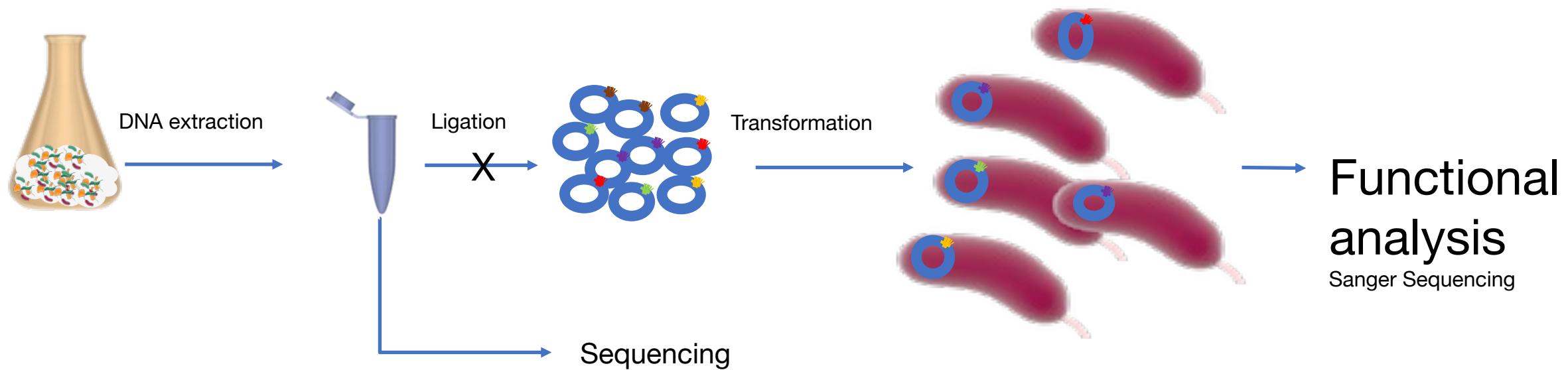
1st generation approach



3. Shotgun metagenomics sequencing

Metagenomics is the technique of retrieving microbial genome directly from the samples

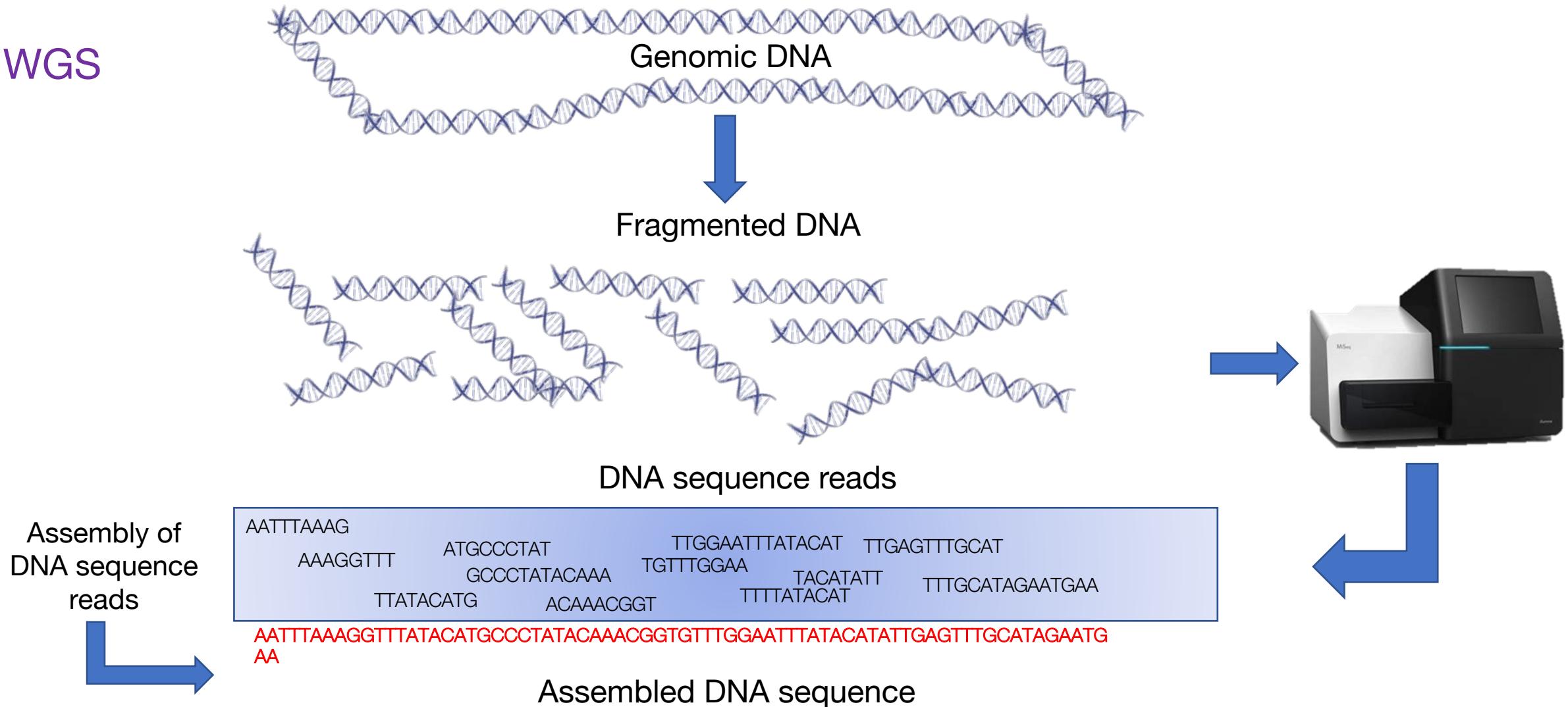
1st generation approach



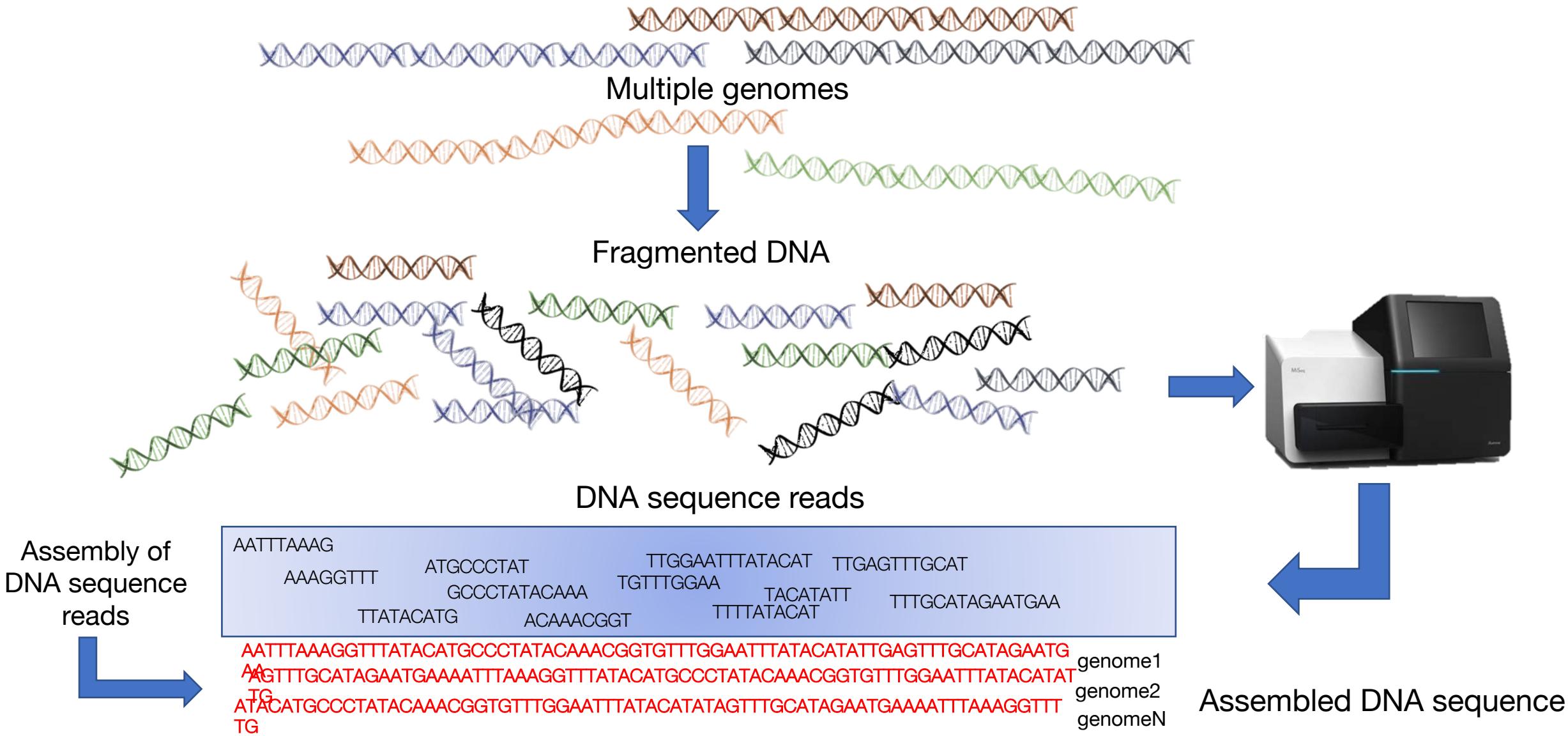
‘sequence-driven metagenomics’

3. Shotgun metagenomics sequencing

WGS



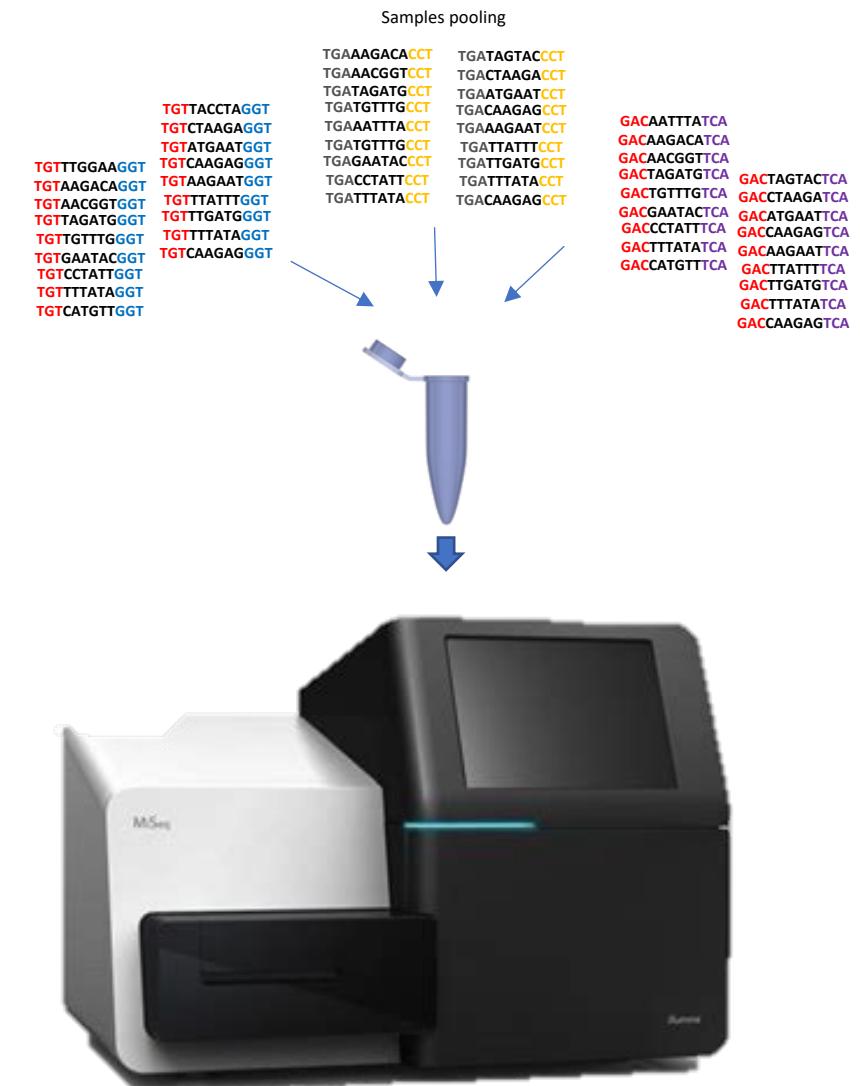
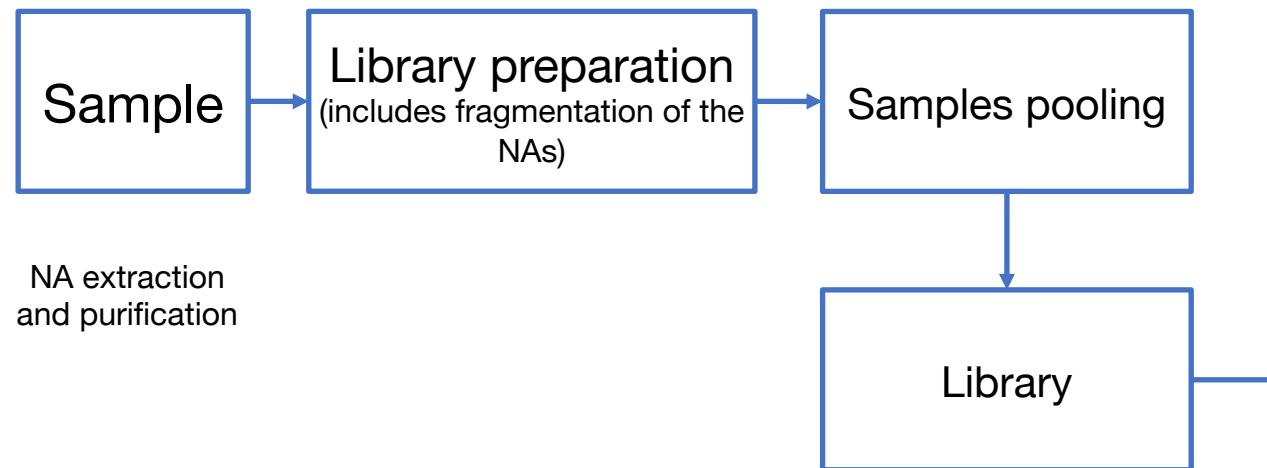
3. Shotgun metagenomics sequencing



3. Shotgun metagenomics sequencing

- Metagenomics is the technique of retrieving microbial genome directly from the samples
- **Explores the entire genetic composition of the microbial communities by sequencing and subsequent analysis**
- **Understanding the biochemical part of the microbes in the atypical environments as well as their interactions with other environmental factors**

3. Shotgun metagenomics sequencing



Extraction and isolation methods can introduce bias in terms of microbial diversity and It's highly recommended that the exact same extraction method be used when comparing sample

Amplification-free based library preparation method is recommended. Certain types of samples (water, swabs) yield small amounts of DNA, necessitating amplification during library preparation.

3. Shotgun metagenomics sequencing

Output analysis

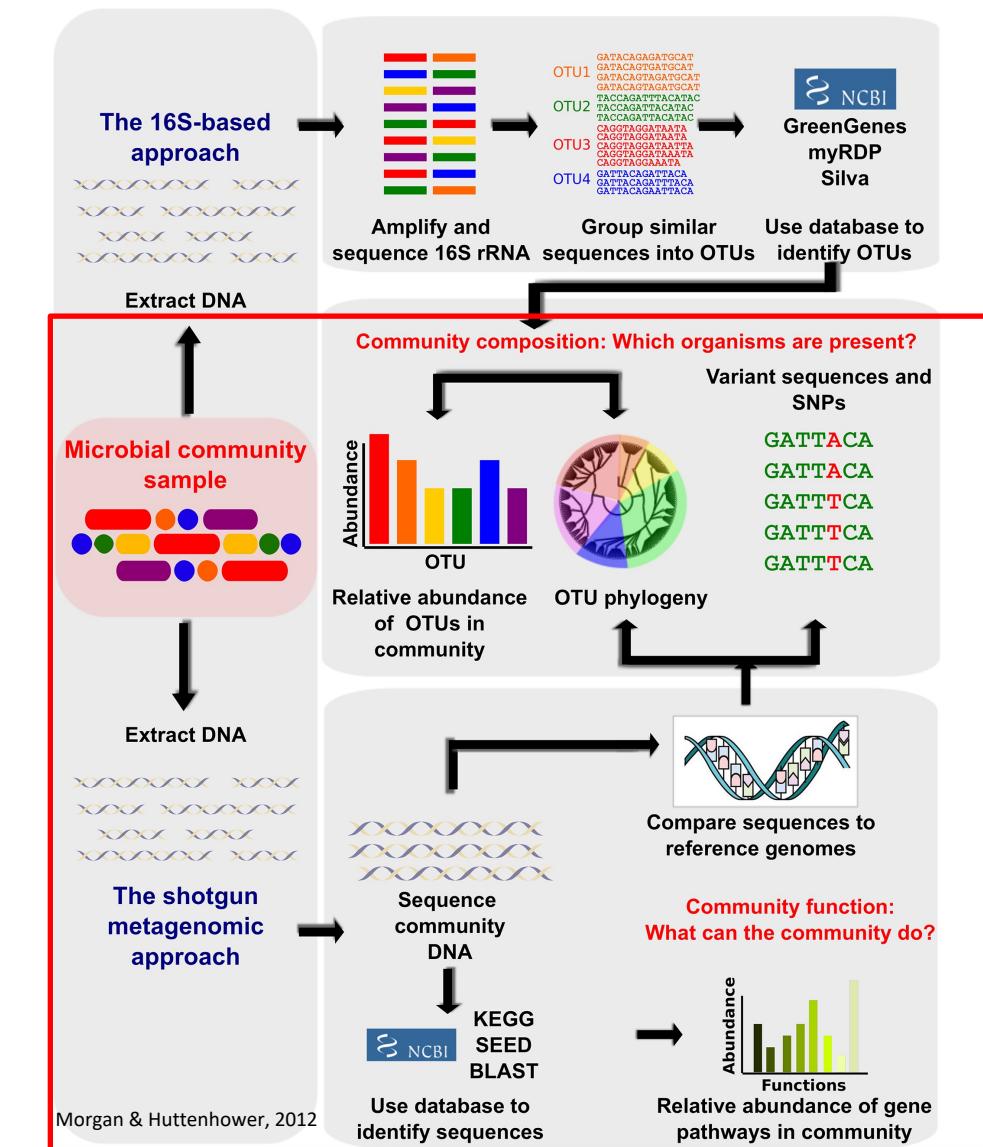
A basic workflow

Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples



3. Shotgun metagenomics sequencing

Output analysis

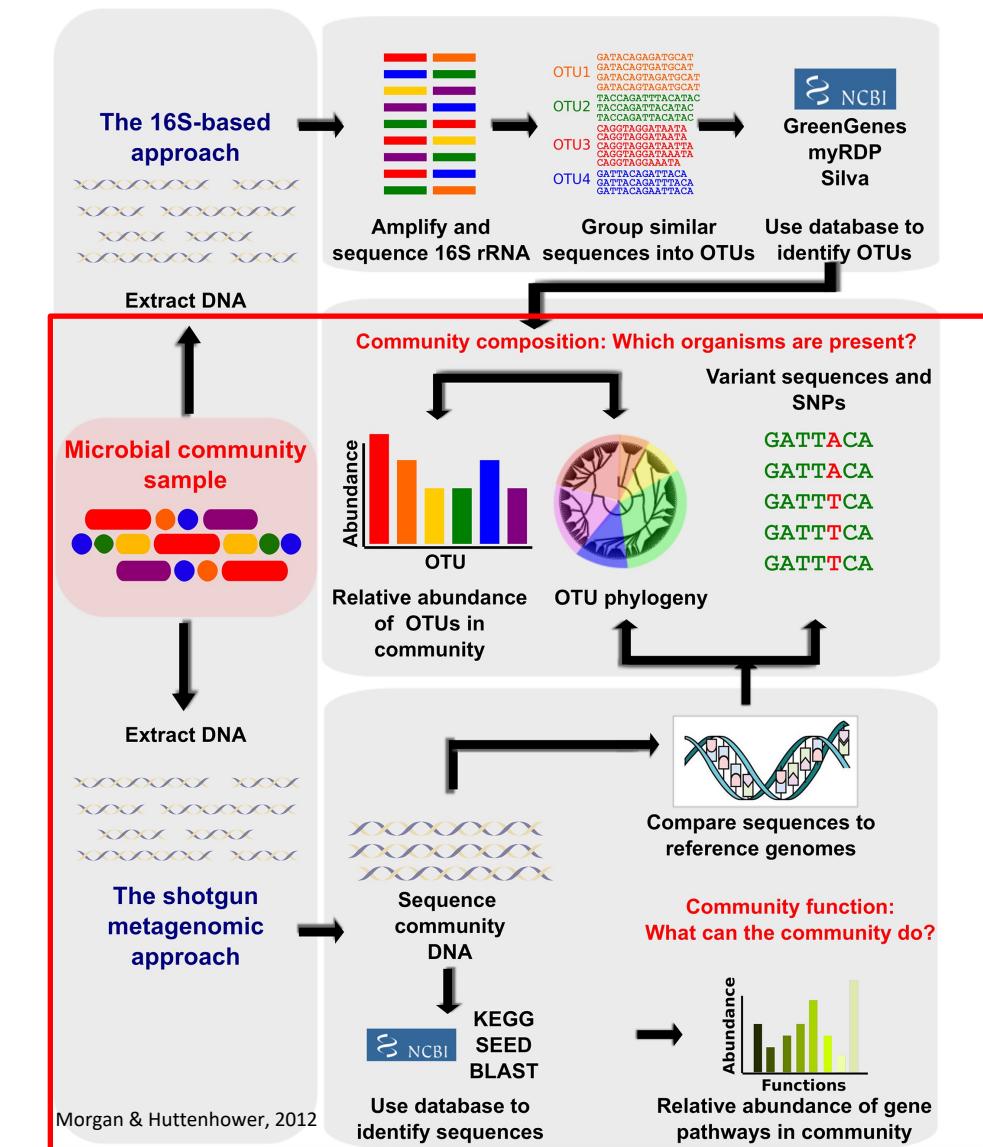
A basic workflow

Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples



3. Shotgun metagenomics sequencing

Output analysis

A basic workflow

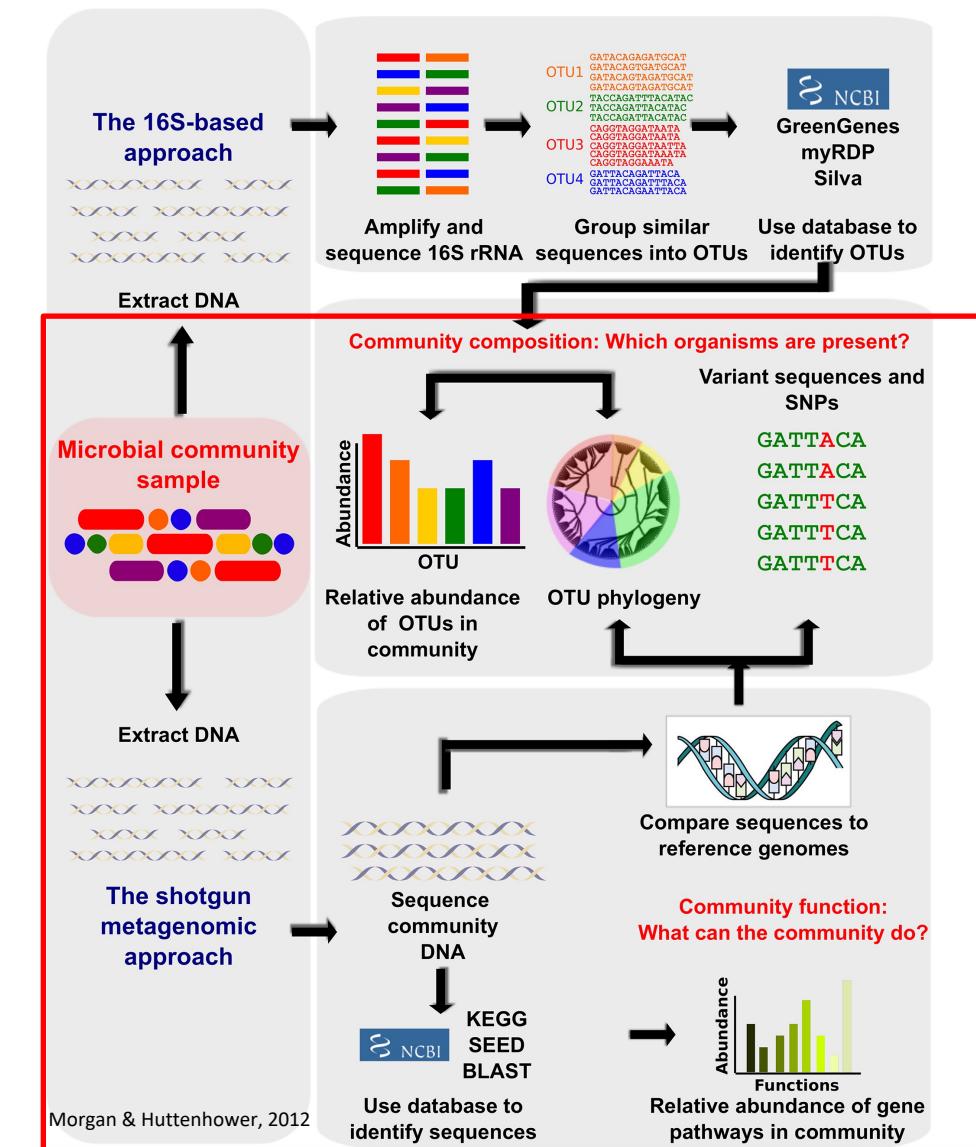
Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples

Binning: the process of grouping reads or contigs and assigning them to OTUs.
 Binning methods can be based on either compositional features or alignment (similarity), or both.



3. Shotgun metagenomics sequencing

Output analysis

A basic workflow

Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples

Similarity-based (reference sequences)

MEGAN: BLAST [Diamond, RapSearch2], LCA algorithm

Mothur & Qiime (16S amplicons): Greengenes, SILVA

MG-RAST (web-server): mainly BLAST

MetaPhyler: phylogenetic marker genes, BLAST

Binning: the process of grouping reads or contigs and assigning them to OTUs.

Binning methods can be based on either compositional features or alignment (similarity), or both.

3. Shotgun metagenomics sequencing

Output analysis

A basic workflow

Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples

Binning: the process of grouping reads or contigs and assigning them to OTUs.

Binning methods can be based on either compositional features or alignment (similarity), or both.

Similarity-based (reference sequences)

MEGAN: BLAST [Diamond, RapSearch2], LCA algorithm

Mothur & Qiime (16S amplicons): Greengenes, SILVA

MG-RAST (web-server): mainly BLAST

MetaPhyler: phylogenetic marker genes, BLAST

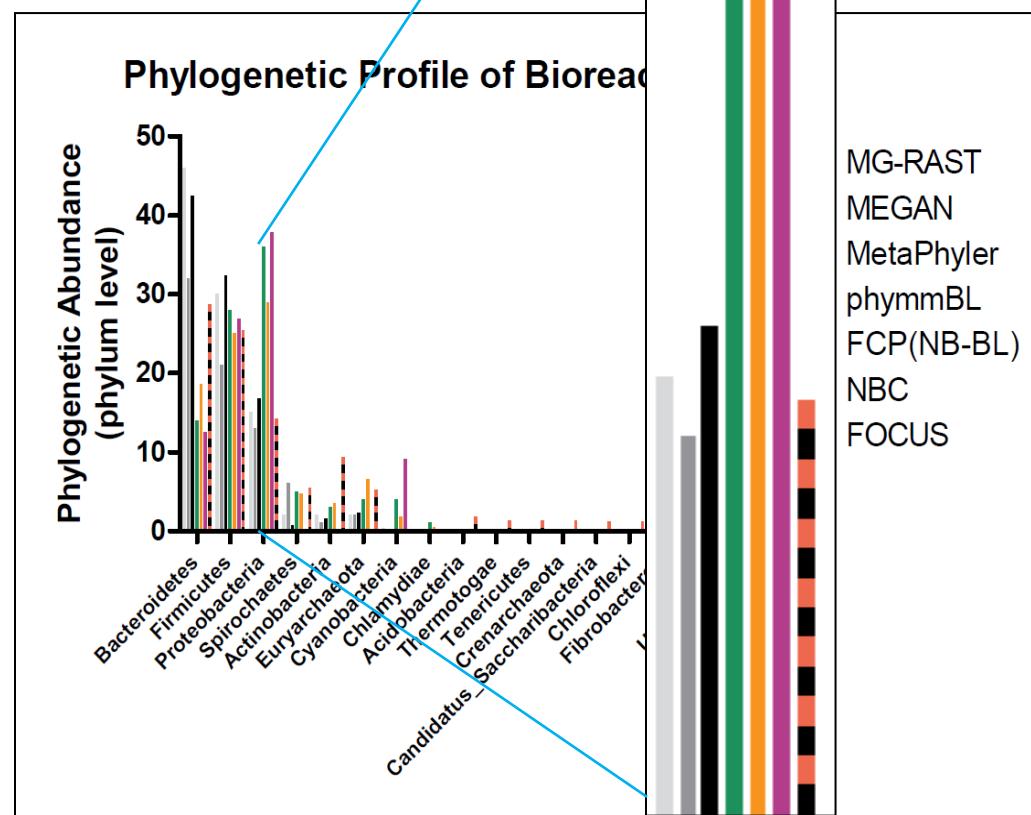
Composition-based (properties of sequences)

- TETRA (tetranucleotide frequency -
<http://www.megx.net/tetra>)
- Naïve Bayesian Classification Tool
(<http://nbc.ece.drexel.edu>)
- PhyloPythiaS (SVMs)
- FOCUS
(<http://sourceforge.net/projects/metagenomefocus/>)

3. Shotgun metagenomics sequencing

Output analysis

A basic workflow



Similarity-based (reference sequences)

MEGAN: BLAST [Diamond, RapSearch2], LCA algorithm

Mothur & Qiime (16S amplicons): Greengenes, SILVA

MG-RAST (web-server): mainly BLAST

MetaPhyler: phylogenetic marker genes, BLAST

Composition-based (properties of sequences)

- TETRA (tetranucleotide frequency - <http://www.megx.net/tetra>)
- Naïve Bayesian Classification Tool (<http://nbc.ece.drexel.edu>)
- PhyloPythiaS (SVMs)
- FOCUS (<http://sourceforge.net/projects/metagenomefocus/>)

3. Shotgun metagenomics sequencing

Output analysis

A basic workflow

Quality control and quality trimming of reads

Binning [classification]

Community profiling

Analyse and compare samples

Relative abundance, heatmaps, ordination, statistical significance

- MEGAN
 - COG, KEGG, SEED
- **HUMAnN software [HMP Unified Metabolic Analysis Network]**
 - uses translated BLAST-vs-KEGG as input
 - collapses hits into gene families/pathways
 - converts to tables of KEGG pathway coverage and abundance
 - tables summarize the gene families and pathways in a microbial community
- **LEfSe [LDA Effect Size] software**
 - visualise HUMAnN output
 - outputs differential features ['biomarkers']

3. Shotgun metagenomics sequencing

| | 16S rRNA (Amplicon Sequencing) | Shotgun Sequencing |
|---|--|---|
| Type of information produced | The taxonomic composition and phylogenetic structure of a microbial community expressed as OTUs ⁸ | Functional and process-level characterization of microbial communities as a whole, and the reconstruction of draft genome sequences for individual community members. |
| Application | Monitor populations | Detect new members, new genes, and resolve complex taxonomies. |
| Ability to detect rare members of the community (sensitivity) | Highly sensitive. rRNA makes up 80% of total bacterial RNA | Requires much deeper sequencing to achieve the same level of sensitivity |
| Biases | Bias produced by the probes ⁹ and the PCR itself ¹⁰ . The amplified region may not accurately represent the whole genome due to horizontal transfer or mutations ¹¹ . | Sequence content bias |
| Gene content | The gene inventory and the encoded functionality of most microbial species are largely unknown and may also vary considerably among strains. | Generate extensive gene inventories and partial genomes. Discover new genes and biological pathways. |

⁸ Operational taxonomic units (OTUs)

⁹ Klindworth et al. 2013 - Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41: e1

¹⁰ Soergel et al., 2012 - Selection of primers for optimal taxonomic classification of environmental 16S rRNA gene sequences. ISME J 6: 1440-1444

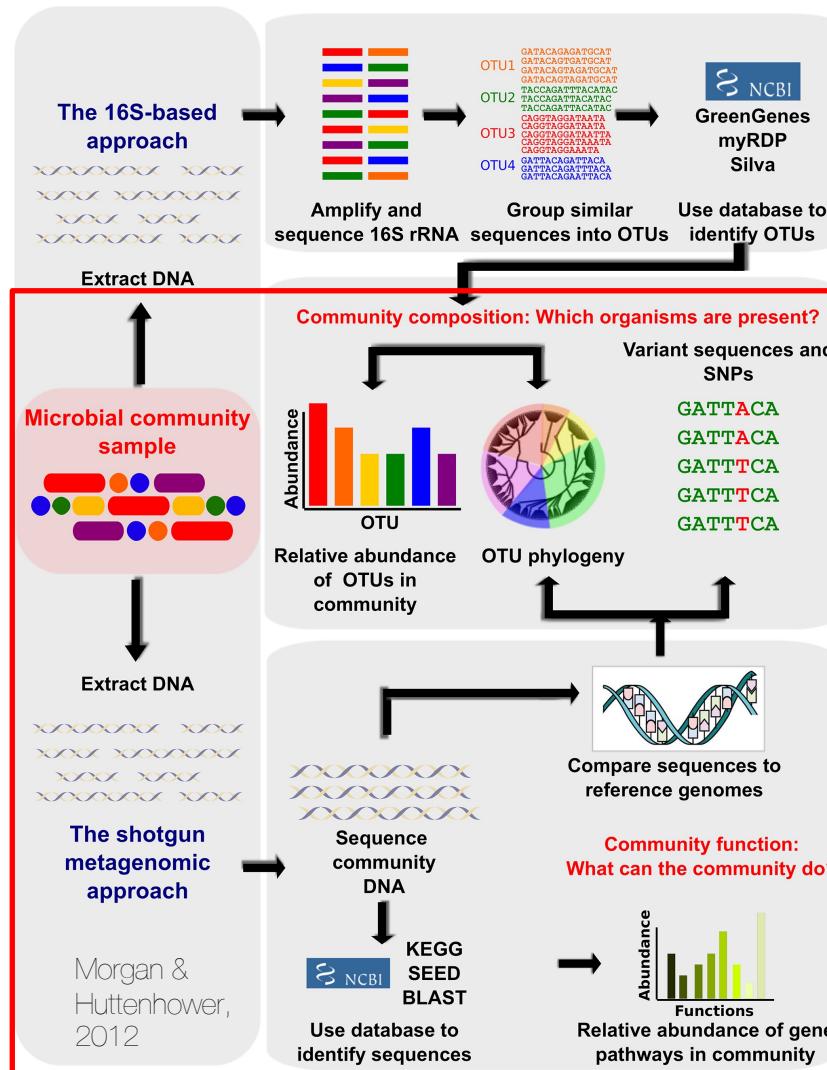
¹¹ Asai et al., 1999 - An Escherichia coli strain with all chromosomal rRNA operons inactivated: complete exchange of rRNA genes between bacteria. Proc Natl Acad Sci U S A 96: 1971-1976

3. Shotgun metagenomics sequencing

| Microbiome | Microbiota | Metagenome |
|---|---|---|
| Microorganisms (and their genes) living in a specific environment | Microorganisms (by type) living in a specific environment | The genes of microorganisms in a specific environment |
| Refers to the bacteria (primary) and their genes (secondary) | Refers to the taxonomy (name) of microorganisms present | Refers to the collective functions of microbial genes |

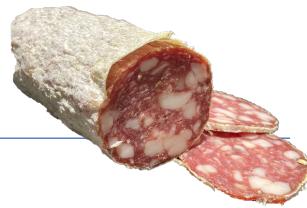
- Introduction to metagenomics
- Amplicon sequencing – 16s rRNA gene sequencing
- Introduction to shotgun metagenomics
- Definitions of microbiome, microbiota and metagenome
- Diversity indexes
- Taxonomy and profiling

4. Microbiomes of fermented foods

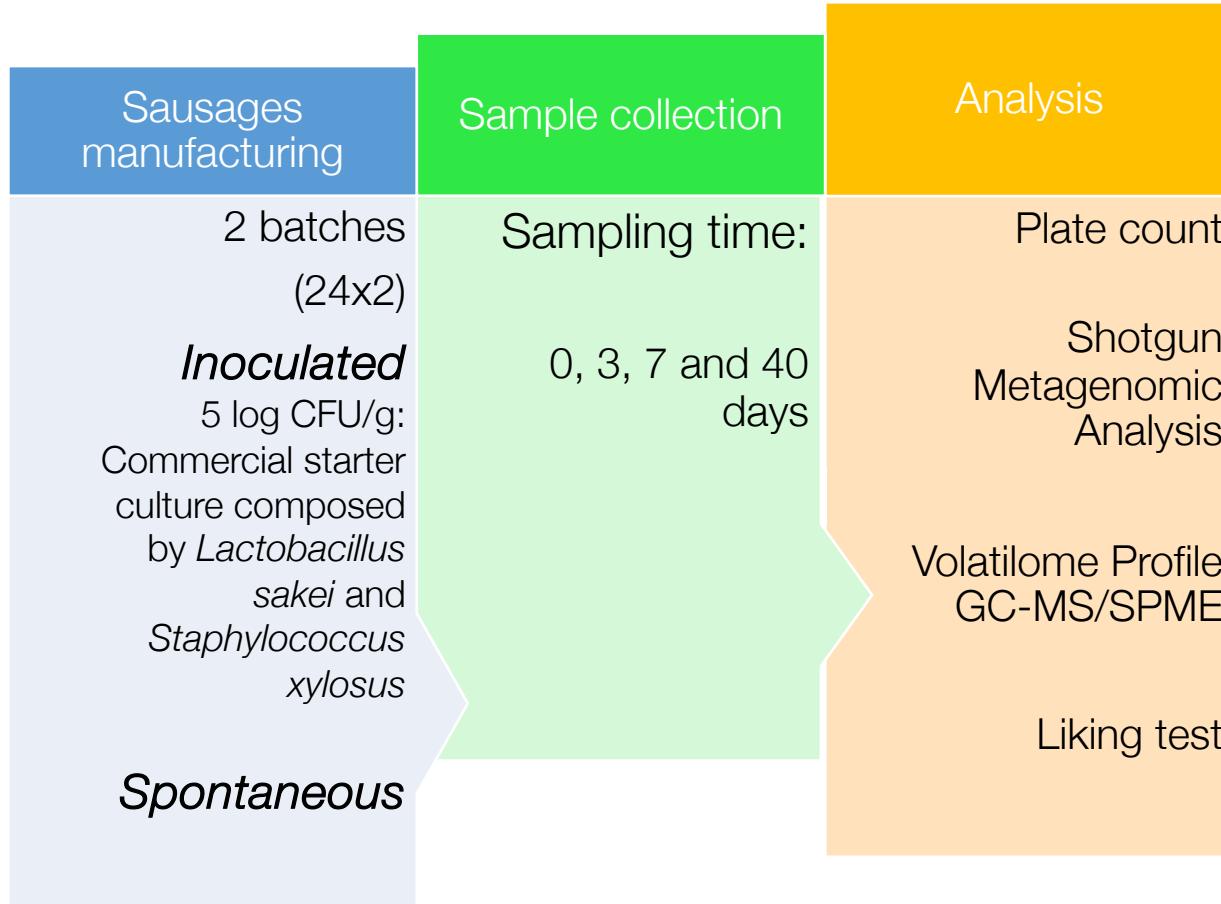


Ferrocino et al., 2017 doi:10.1128/AEM.00983-17

4. Microbiomes of fermented foods



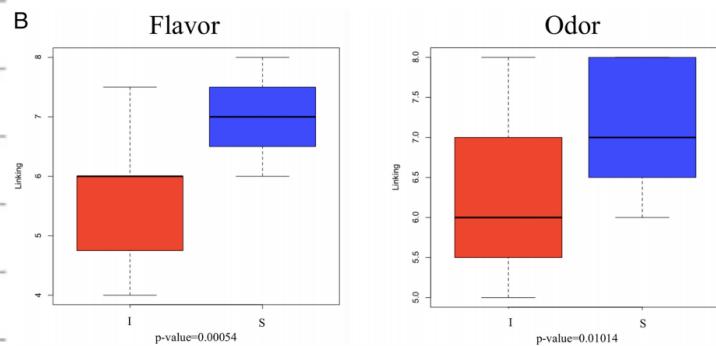
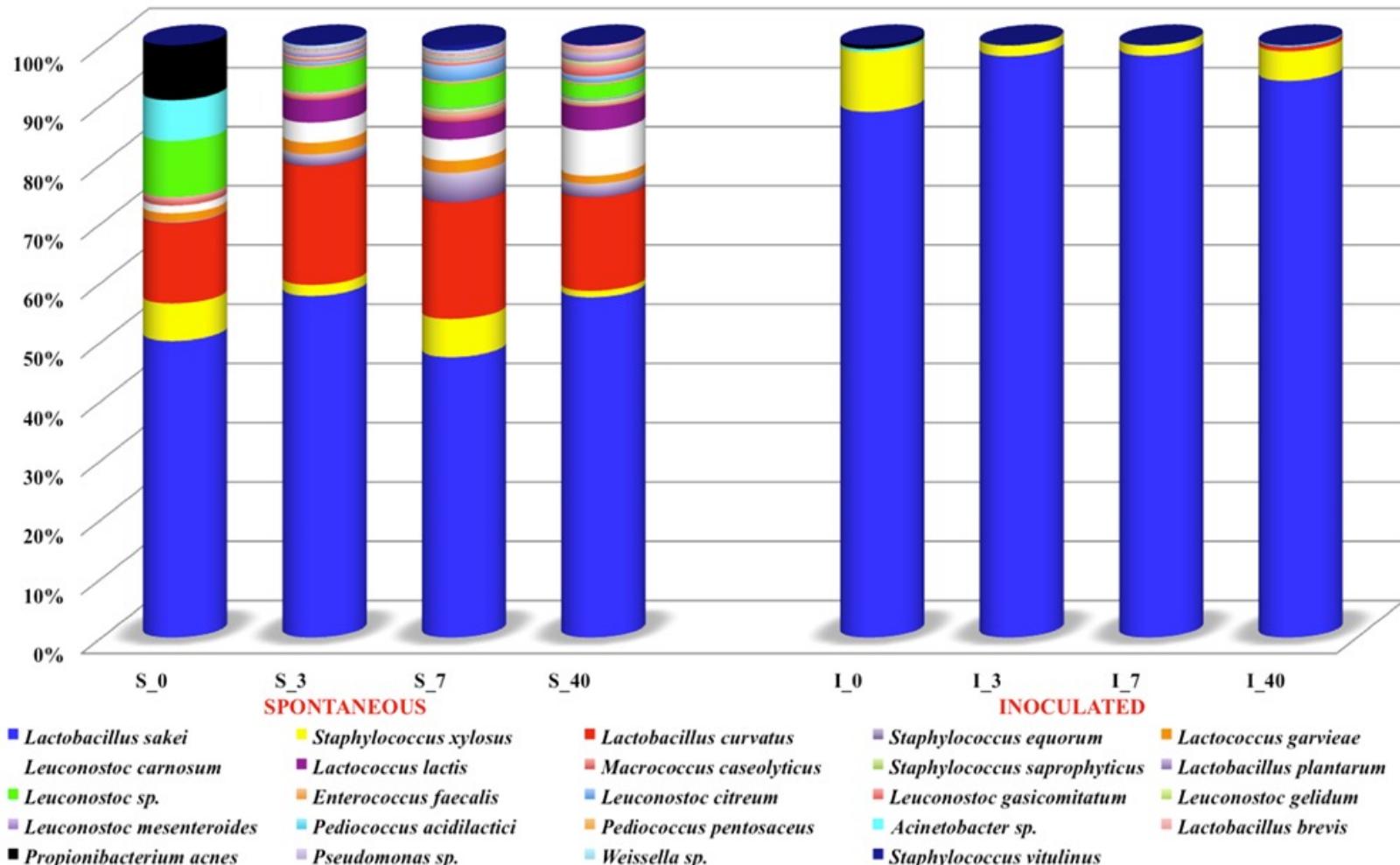
| Step | Time (h) | Temperature (°C) | Relative humidity (%) |
|--------------|--------------|------------------|-----------------------|
| dry | 8 | 20-22 | 92-95 |
| fermentation | 24 | 19-21 | 80-88 |
| | 24 | 19-21 | 72-84 |
| | 24 | 18-20 | 68-76 |
| | 24 | 17-19 | 72-78 |
| | 24 | 16-18 | 74-82 |
| | 24 | 15-17 | 70-78 |
| ripening | till 40 days | 12-14 | 74-88 |



Ferrocino et al., 2017 doi:10.1128/AEM.00983-17

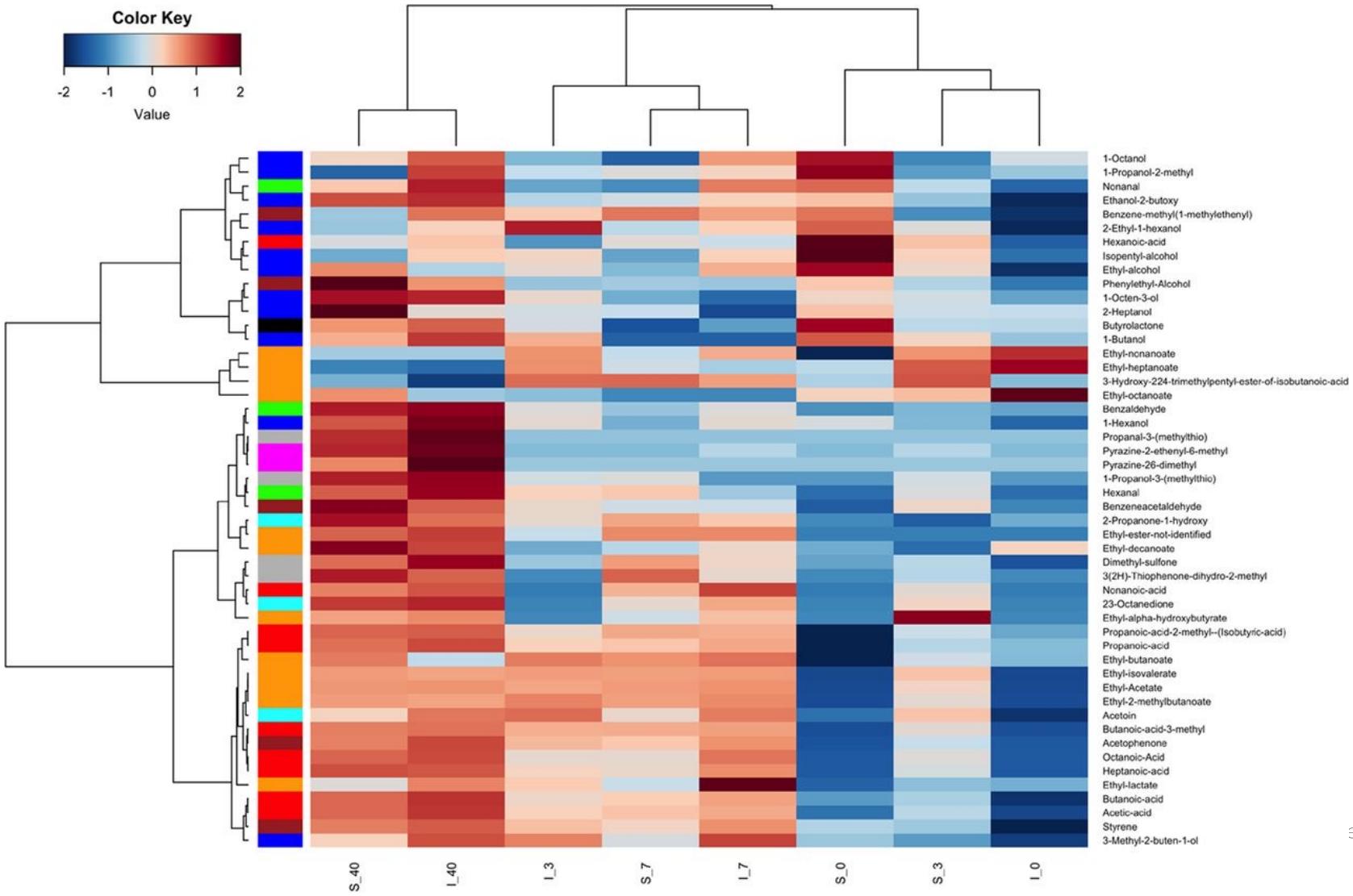
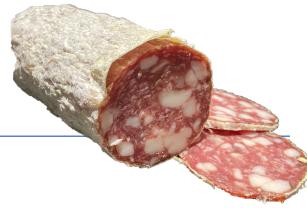


4. Microbiomes of fermented foods



Ferrocino et al., 2017 doi:10.1128/AEM.00983-17

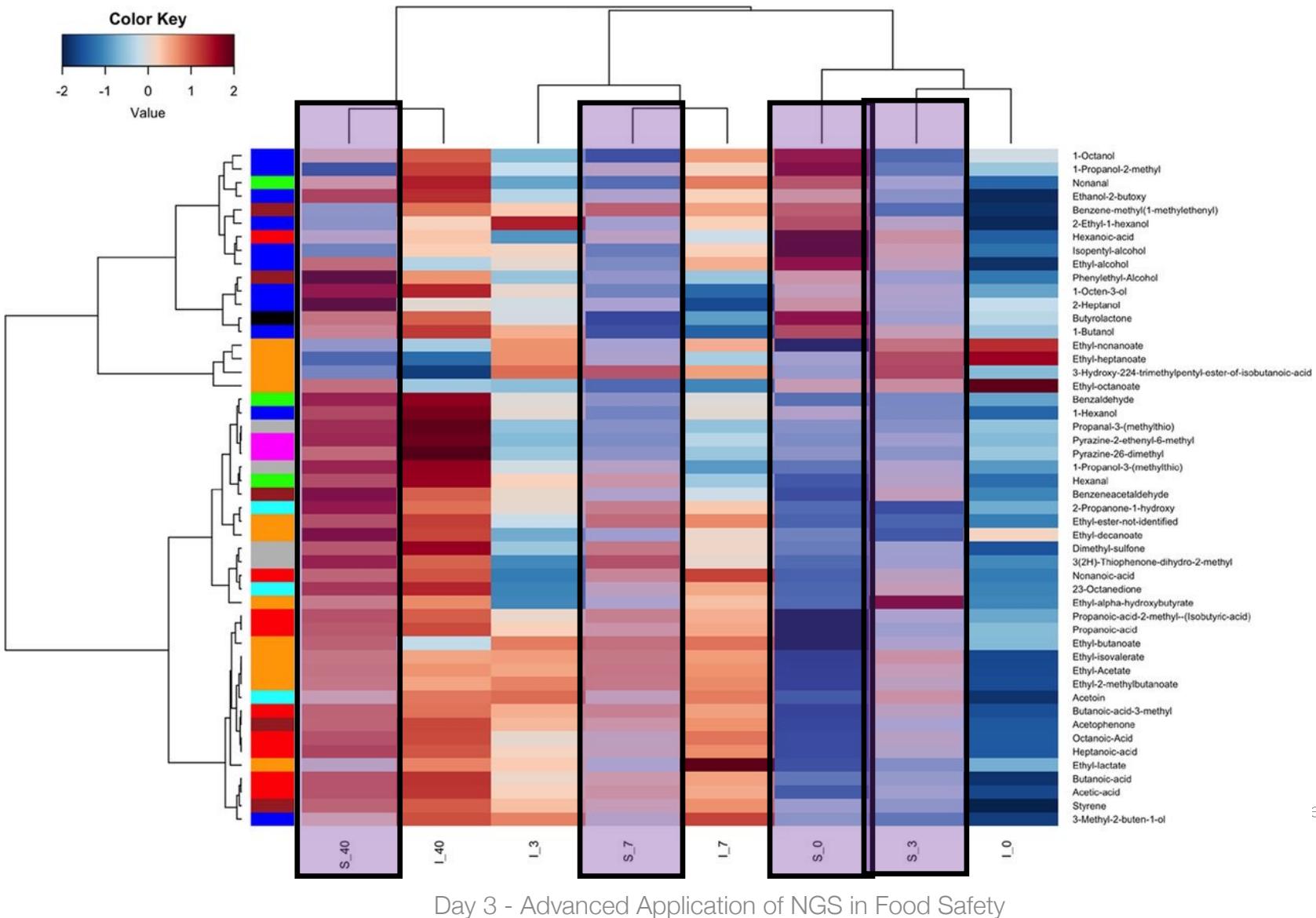
4. Microbiomes of fermented foods



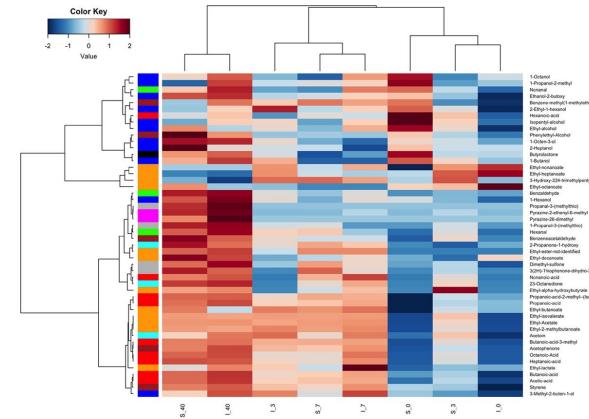
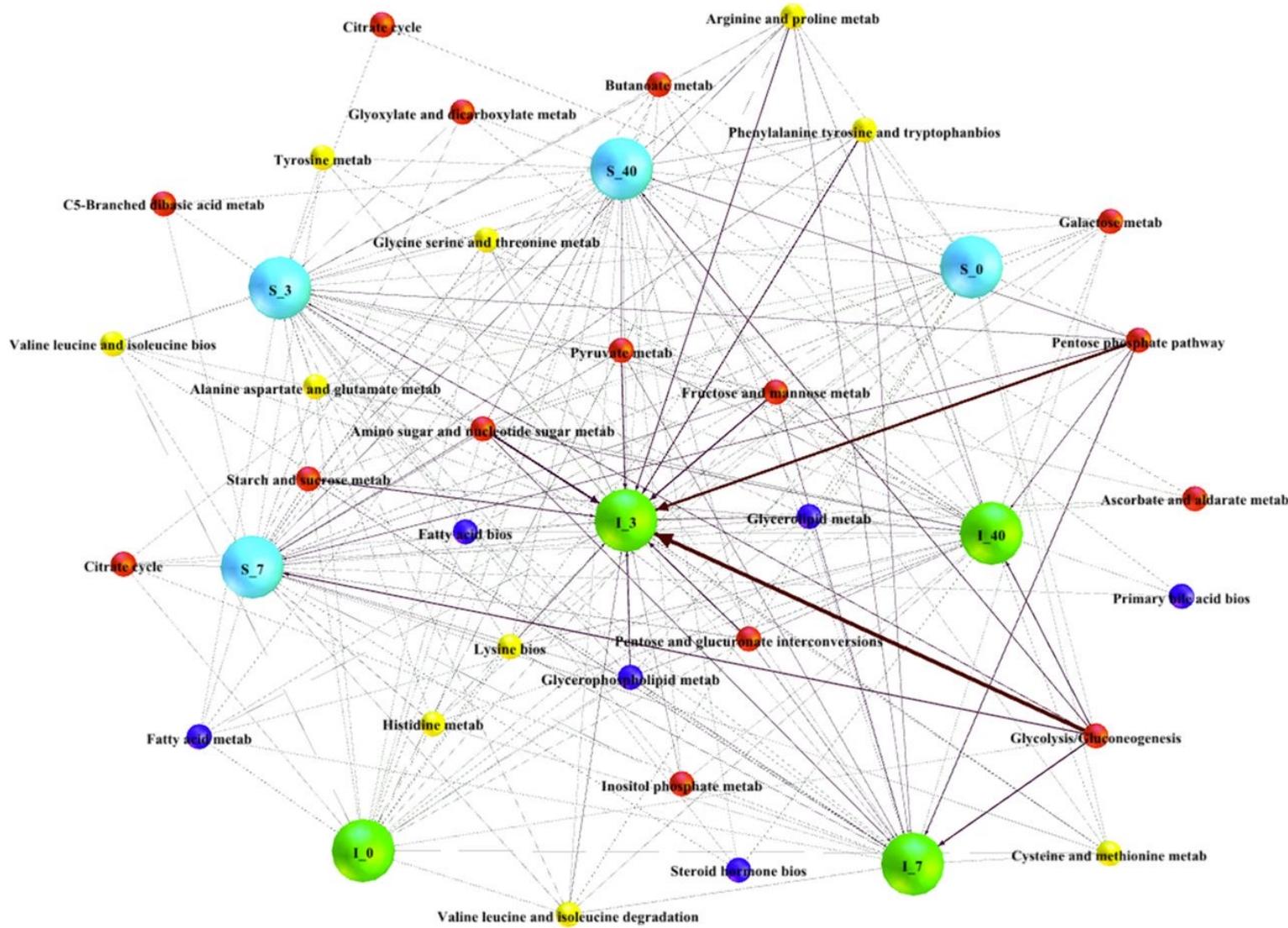
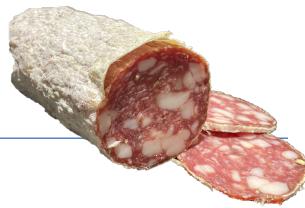
erricino et al., 2017 doi:10.1128/AEM.00983-17



4. Microbiomes of fermented foods



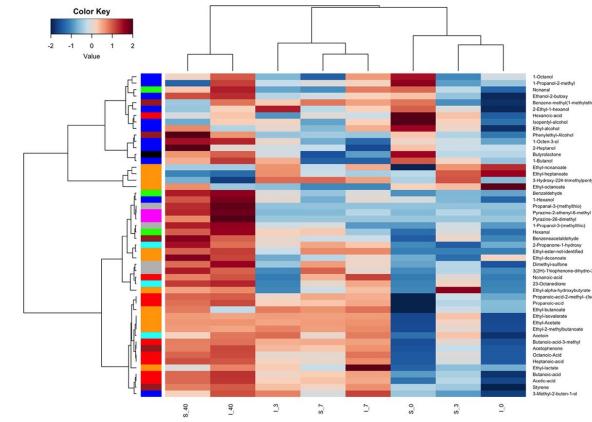
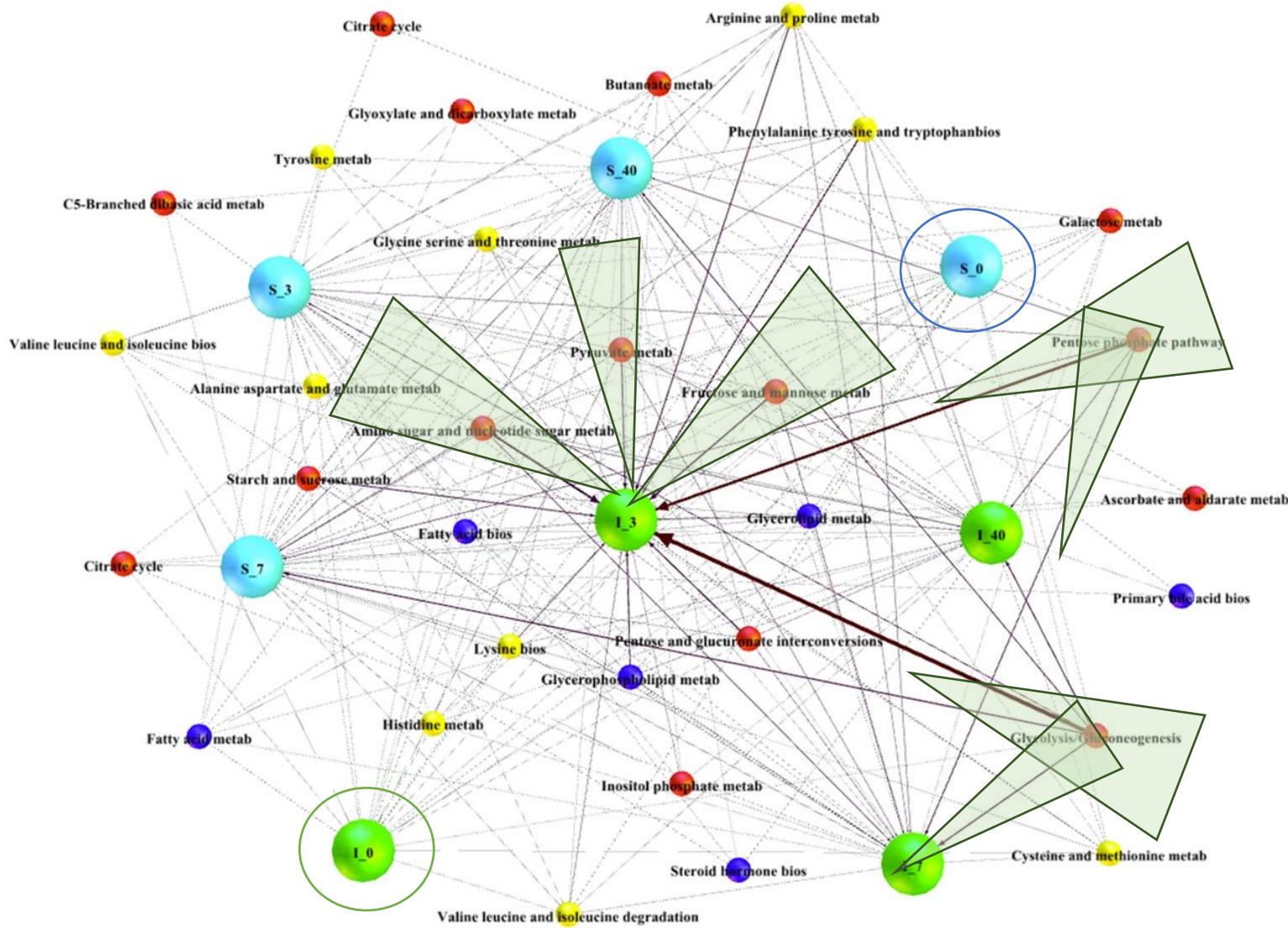
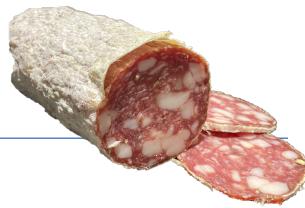
4. Microbiomes of fermented foods



Ferrocino et al., 2017 doi:10.1128/AEM.00983-17



4. Microbiomes of fermented foods

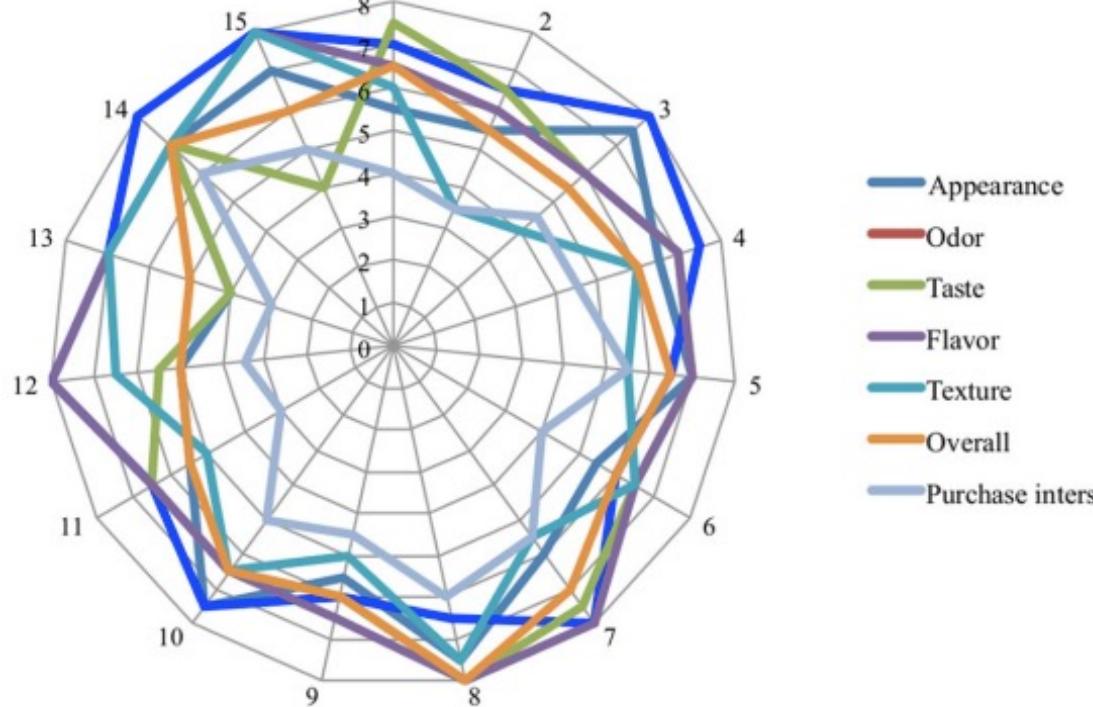


Ferrocino et al., 2017 doi:10.1128/AEM.00983-17

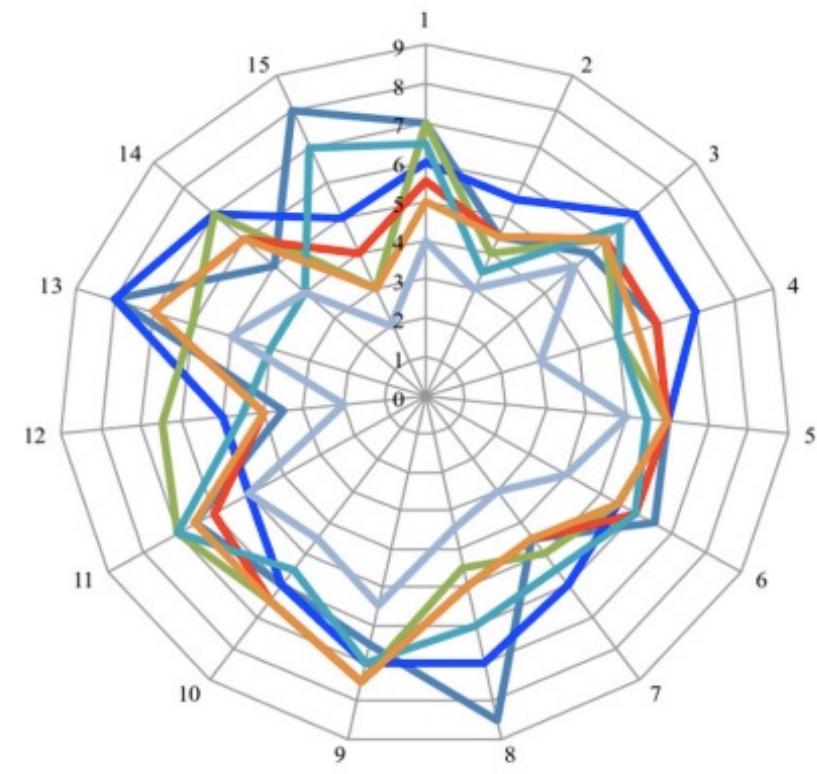
4. Microbiomes of fermented foods



Spontaneous

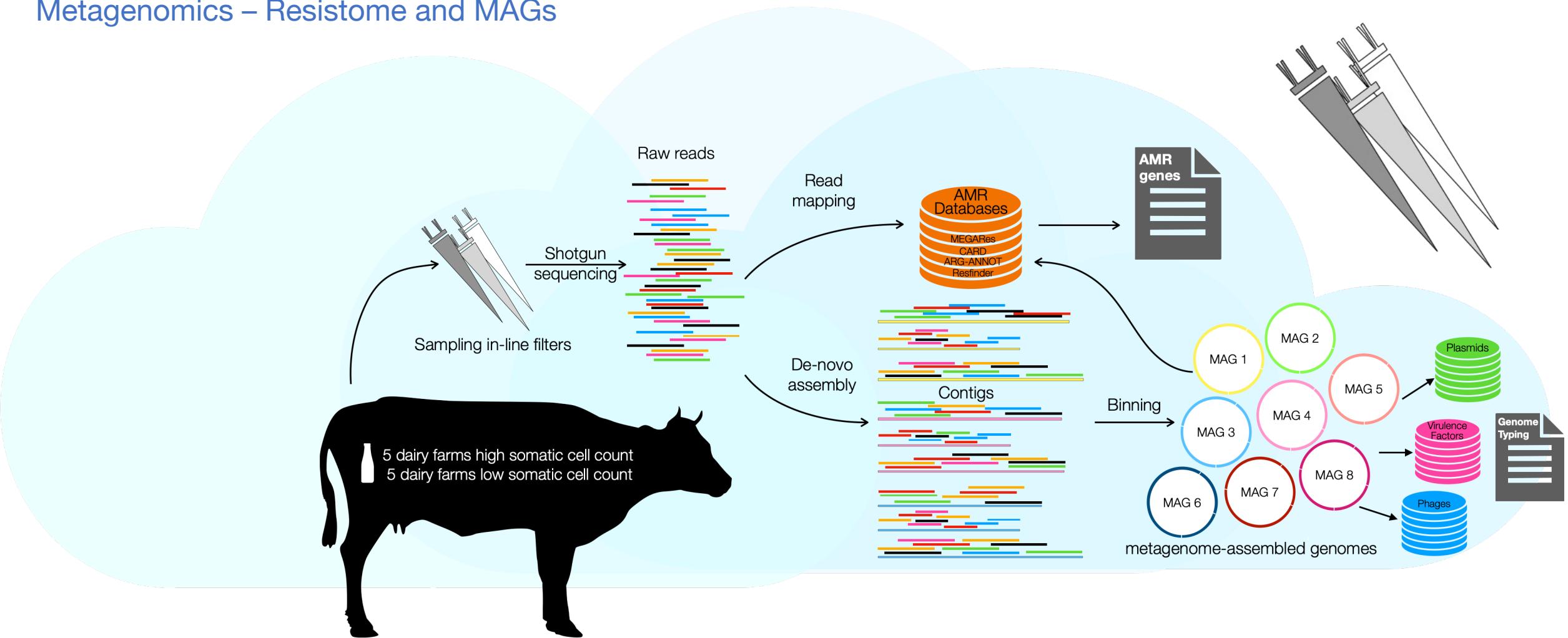


Inoculated



5. Resistome

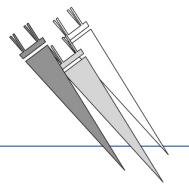
Metagenomics – Resistome and MAGs



Rubiola et al., 2022 doi:10.1016/j.foodres.2022.111579

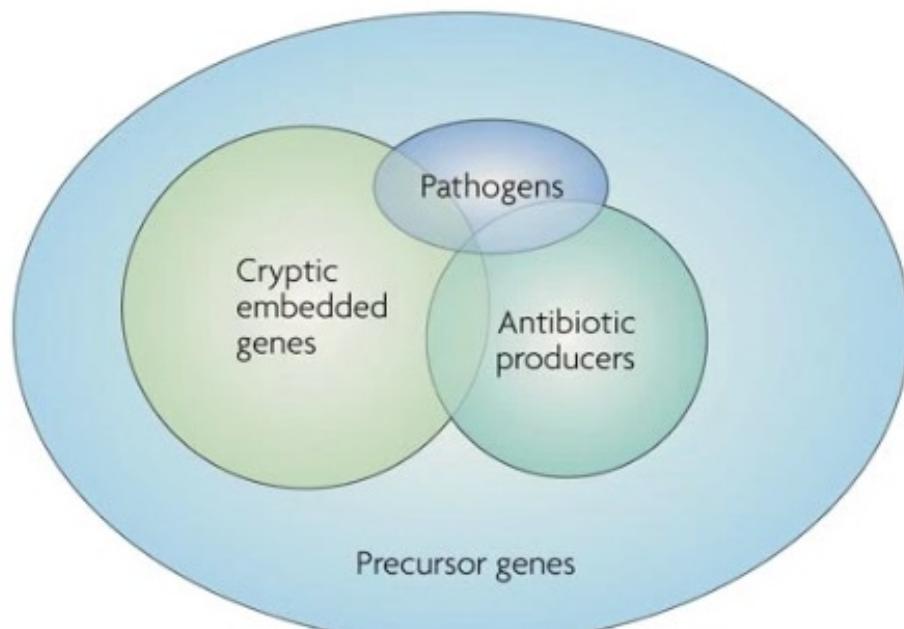


5. Resistome



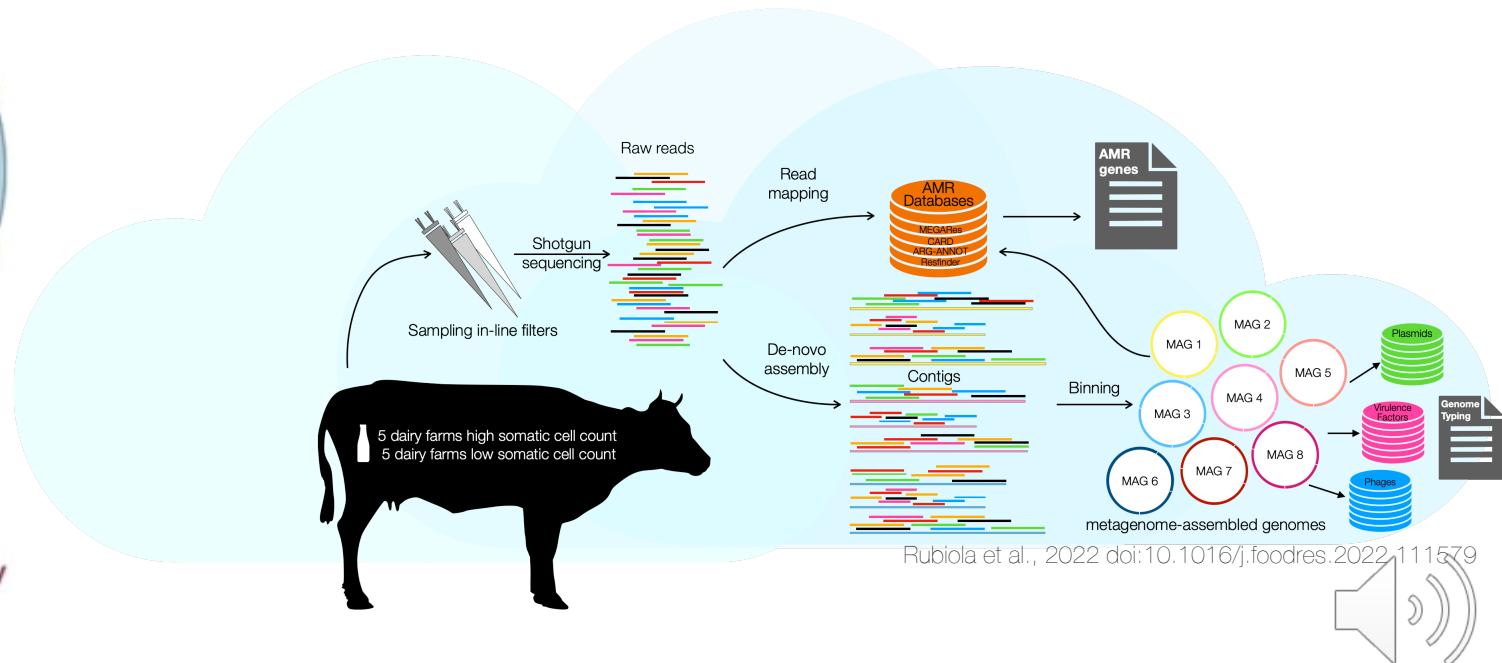
Metagenomics – Resistome and MAGs

The resistome **comprises all of the antibiotic resistance genes**. It includes resistance elements found in both pathogenic bacteria and antibiotic-producing bacteria, and cryptic resistance genes (which are not necessarily expressed) that are present in bacterial chromosomes.



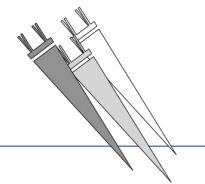
Wright 2007 doi:10.1038/nrmicro1614

Nature Reviews | Microbiology

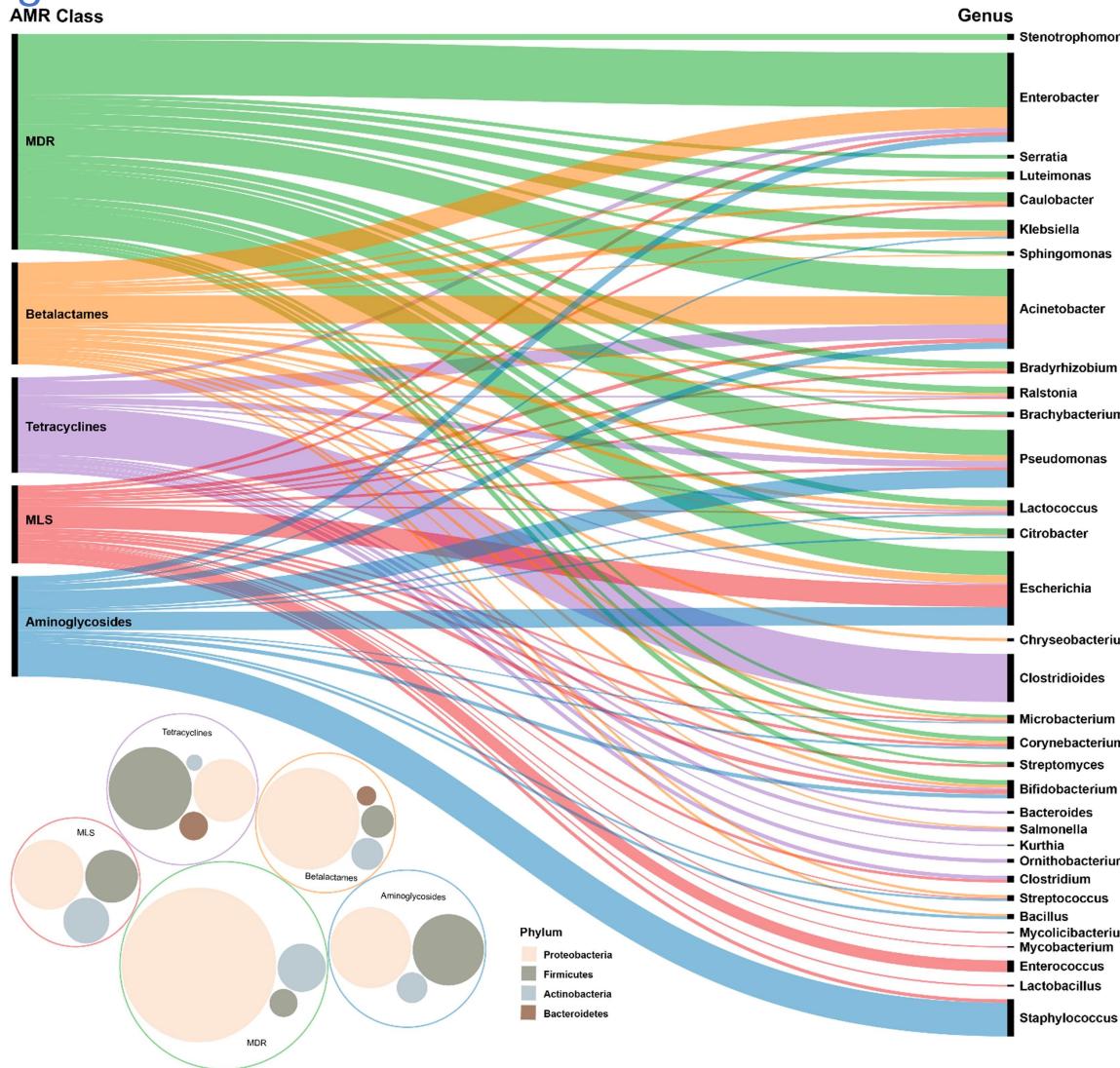


Rubiola et al., 2022 doi:10.1016/j.foodres.2022.111579

5. Resistome



Metagenomics – Resistome and MAGs

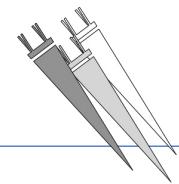


- Reads-based and assembly-based approaches allowed the identification of AMR genes.
- *Enterobacter, Acinetobacter, Escherichia* and *Pseudomonas* harboured most of AMR genes.
- Risk associated with poor hygiene practices in the dairy production environment and consumers of the potential microbial food safety risks derived from raw milk products consumption

Rubiola et al., 2022 doi:10.1016/j.foodres.2022.111579

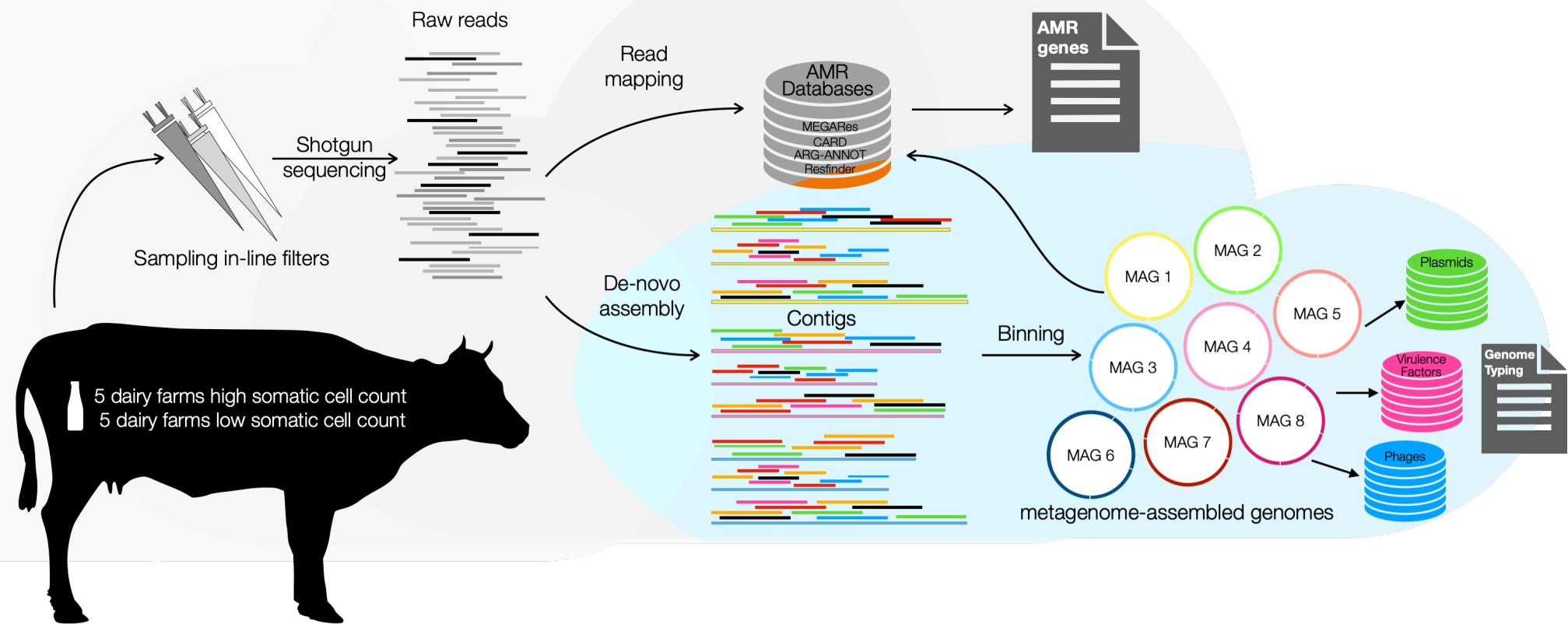


5. Resistome



Metagenomics – Resistome and MAGs

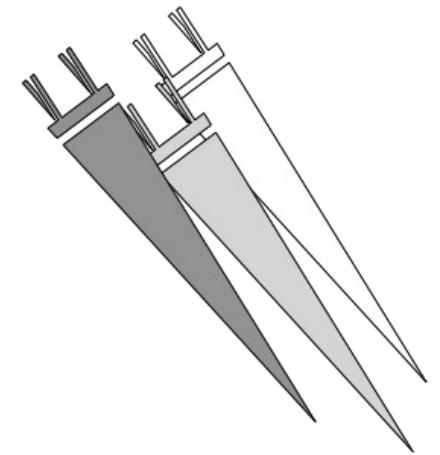
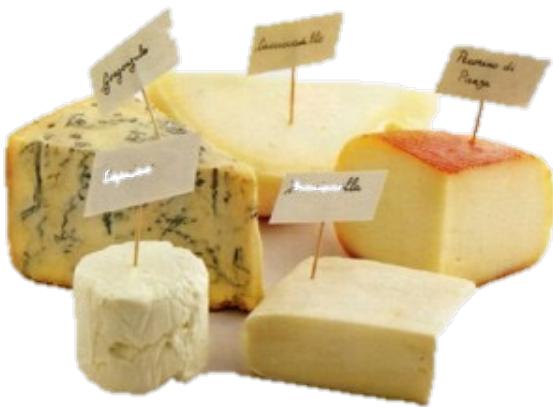
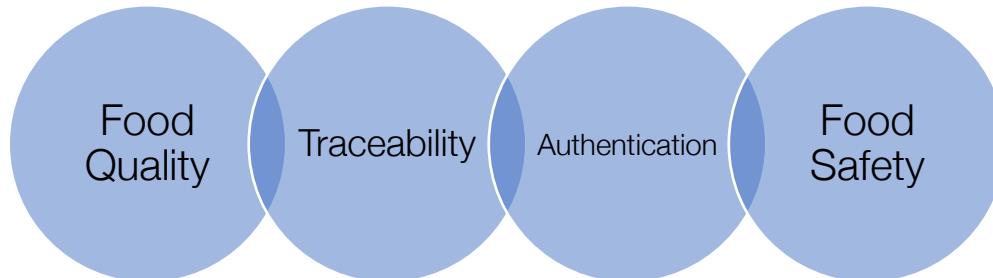
Metagenome-Assembled Genomes MAGs – it is a single-taxon assembly based on one or more binned metagenomes that has been asserted to be a close representation to an actual individual genome (that could match an already existing isolate or represent a novel isolate).



Rubiola et al., 2022 doi:10.1016/j.foodres.2022.111579

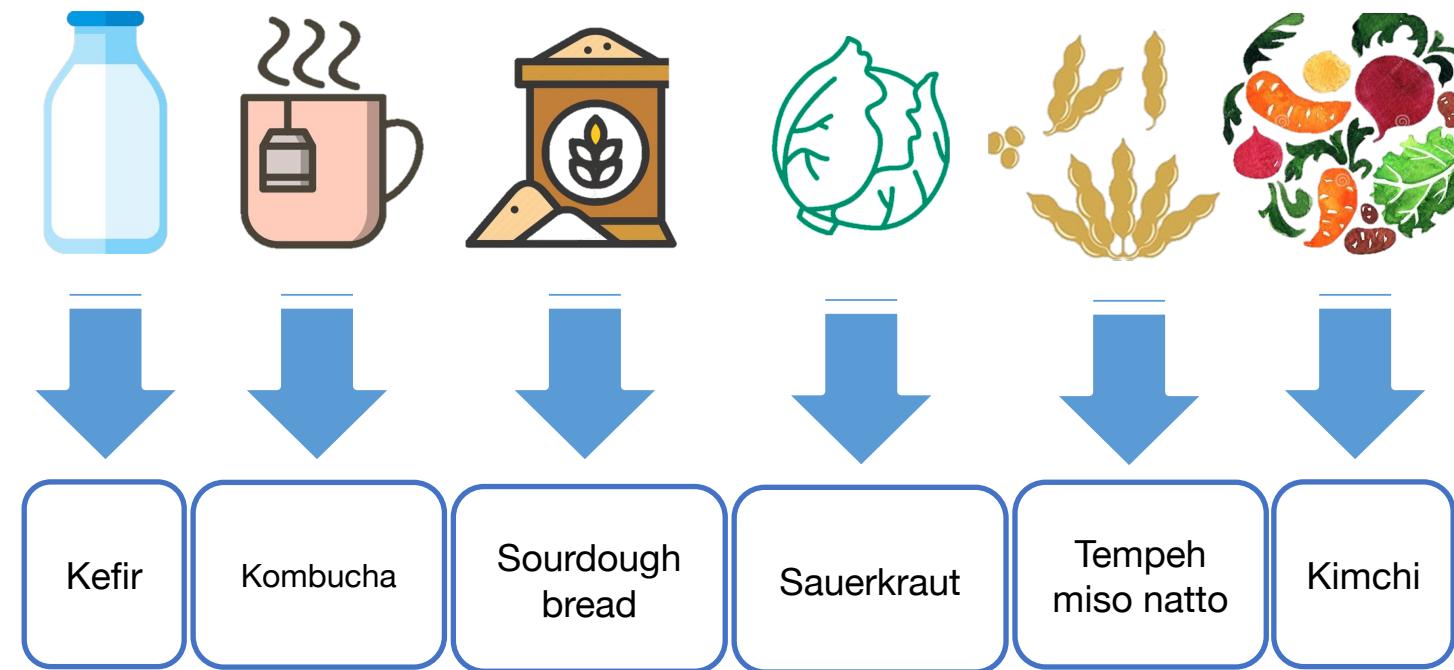
6. Food microbiomes

next generation of food safety controls

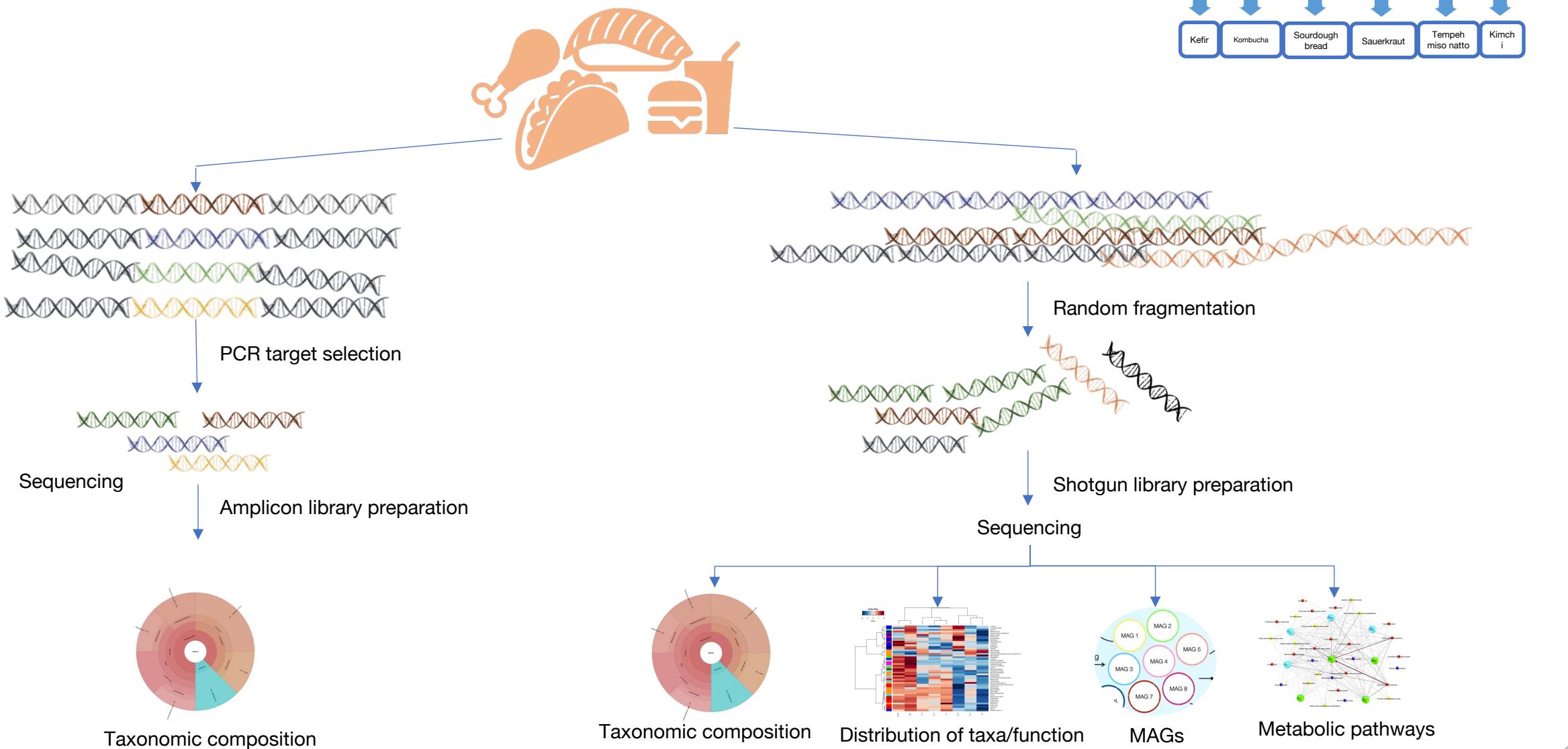


- WGS
- Metagenomics (16s rRNA gene sequencing/amplicon and shotgun sequencing)
- Genomes of microbiomes

6. Food microbiomes



6. Food microbiomes – a recap



- Application metagenomics food safety and food quality
- Resistome
- Food microbiome
- Human microbiome
- The microbiome of fermented foods, can we hack our gut microbiome?

