

Introduction to the course:

Microbiology of Fermented Foods and Beverages (MFFB)

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Content

- **Fermented foods and their microorganisms – in introduction**
- Learning outcomes
- Groups and groupwork – food products to work on
- Teachers and technicians
- Laboratory exercises, exercises, cases
- Project presentations by student teams
- Exam
- Safety in the laboratory

Dinner invitation – please enjoy!



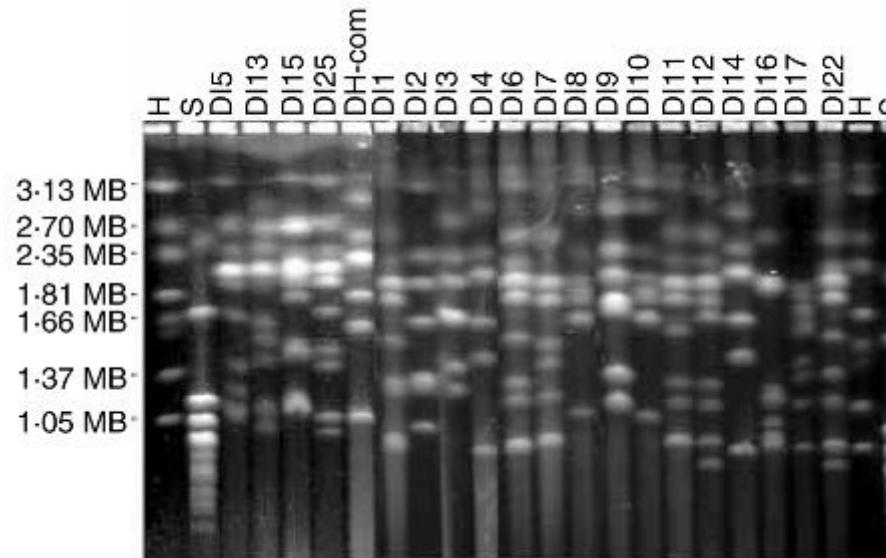
Advantages obtained by fermentation

- Improved organoleptic and textural quality
 - Production of aroma compounds (esters, alcohols, organic acids, carbonyls a.o.)
 - Ethanol production !
 - Production of tissue degrading enzymes (cellulases and pectinases)
- Prolonged shelf life and improved safety
 - Inhibition of pathogens and spoilage organisms (production of antimicrobials etc.)
 - Reduced loss of raw materials (sustainable production)
 - Reduced cooking time (energy saving)
- Health beneficial effects
 - Improvement of bio-availability of micronutrients
 - Improvement in protein quality, carbohydrate digestibility and vitamin synthesis
 - Reduction/elimination of toxic or anti-nutritional factors (e.g. phytase and linamarase activity or binding/degradation of mycotoxins)
 - “Probiotic properties”



Starter culture development for semi-hard cheeses

"Indigenous" dairy cultures – **strain variations** of *D. hansenii* strains



⇒ ***Debaryomyces hansenii* is a very heterogenous species**

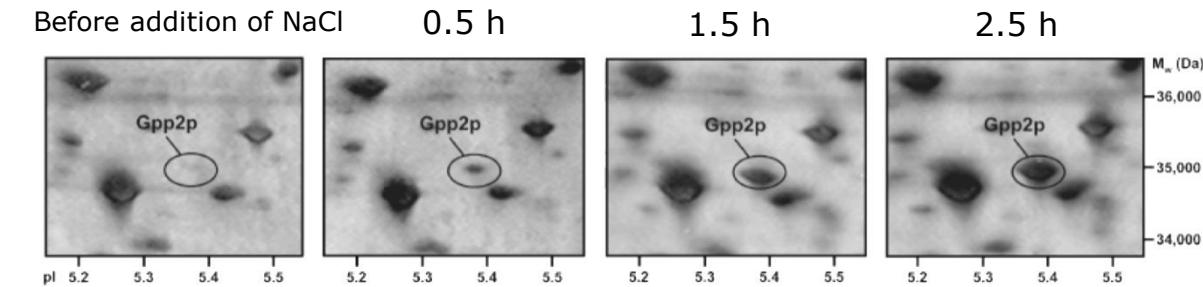
For the haploid CBS767 the total genome size could be estimated to approx. 11.9 Mb – six chromosomes

Danish surface ripened cheeses



A superior stain of *D. hansenii* was developed and implemented as starter culture at the industrial level

exposure to 8% (w/v) NaCl



☞ AL436338 = *DhGPP2*

Let's drink – innovation and microbial teamwork

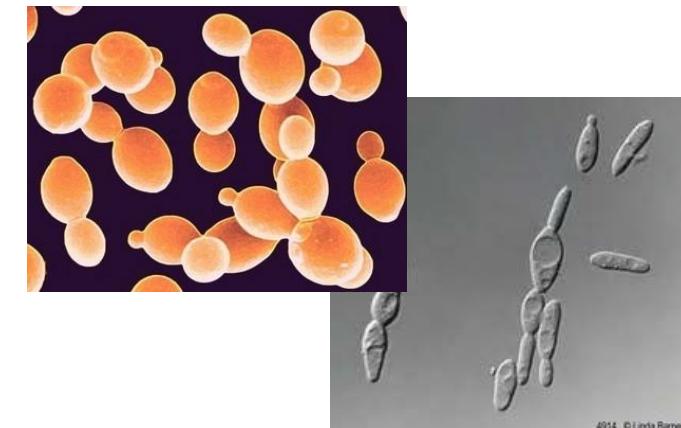
Alulu beer recipe – 3200 BC, Babylon



The microbial (r)evolution in the beverage industry:
from indigenous wild largely uncontrolled
fermentation to purified starter culture – and back!



Beer made with a combination of *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis*



Fermentation for scientists..

McGee, H. (2013) A festive ferment. **Nature** 504, 372-374

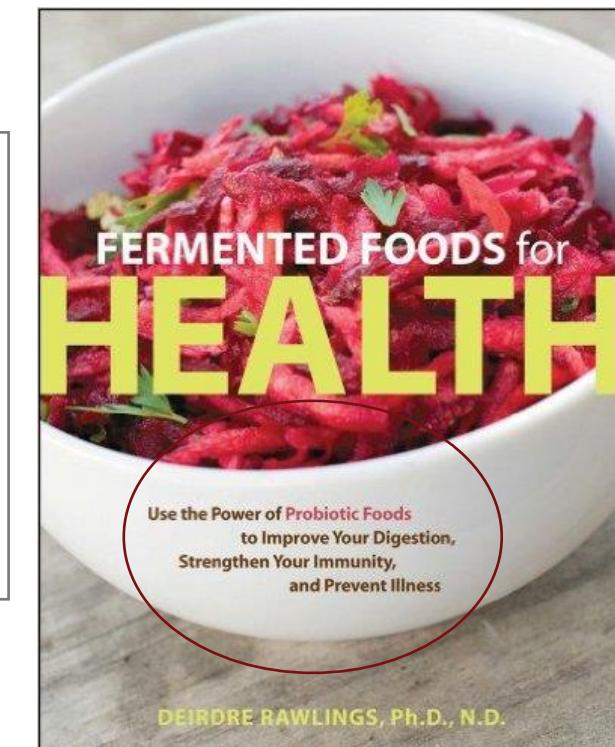


Delicacies in which fermentation plays a part include, left to right: pickled cucumber; salami; chocolate; Korean kimchi, or pickled cabbage; Japanese natto, or fermented soya beans; and soy sauce.

"Rare is the holiday meal that does not owe many of its pleasures to invisible cooks with tongue-twisting names. Do you enjoy charcuterie and pickles? Bread with cultured butter? A drizzle of vinaigrette on this or that? A bit of cheese? Some chocolates? Wine, beer or cider? Then raise a glass to *Saccharomyces cerevisiae*, *Leuconostoc mesenteroides* and their ilk, the fungi and bacteria that do the real work of... turning blandness into piquant delight".

Food microbiology
is rocket science!

... and others!



Learning outcomes MFFB (NFOK14019U) 1/3

Knowledge:

Theoretical or practical understanding of...

- Show overview of **fermented food and beverages** in general and the microorganisms involved in their production
- Describe important **groups of microorganisms** identified from fermented food and beverages
- Comprehend microbial **taxonomic systems**
- Reflect on microbial **cytology and physiology**
- Describe microbial **interactions** and their importance in food systems
- Define **molecular techniques for identification and typing** to species and strain level

Learning outcomes MFFB (NFOK14019U) 2/3

Skills:

Have the ability to…

- **develop procedures and plans for isolation and identification of the predominant microorganisms in fermented food and beverages**
- **explain at the molecular level the behavior and interactions between various groups of microorganisms**
- **identify the most important parameters leading to optimal product quality and food safety**
- **apply food fermentation to develop innovative food products**

Learning outcomes MFFB (NFOK14019U) 3/3

Competencies:

Being able to do...

- Isolation of microorganisms from different types of fermented food and beverages
- Evaluation of the composition of the microbiota of fermented food and beverages
- Identification of the predominant microorganisms by both phenotypic and genotypic methods
- Description of presumed functionalities of microorganisms in fermented food or beverages

Teaching periods

- We are in Block 1
 - Confrontation hours (approx.):
 - Monday: 13-17
 - Wednesday: 9-17
 - In addition, one day per week for preparation to the course

Lecturers and technicians

- Lene Jespersen (LJ, responsible)
- Dennis S. Nielsen (DN)
- Nils Arneborg (NA)
- Henrik Siegumfeldt (HS)
- Nadja Larsen (NL)
- Lukasz Krych (LK)
- Paulina Deptula



- Tessay Canoy (TC)



- Henriette Lyng Røder



- Bashir Aideh (BAI)



- Denitsa Stefanova (DS)



- Marina Kryger Bjørklund



Course content

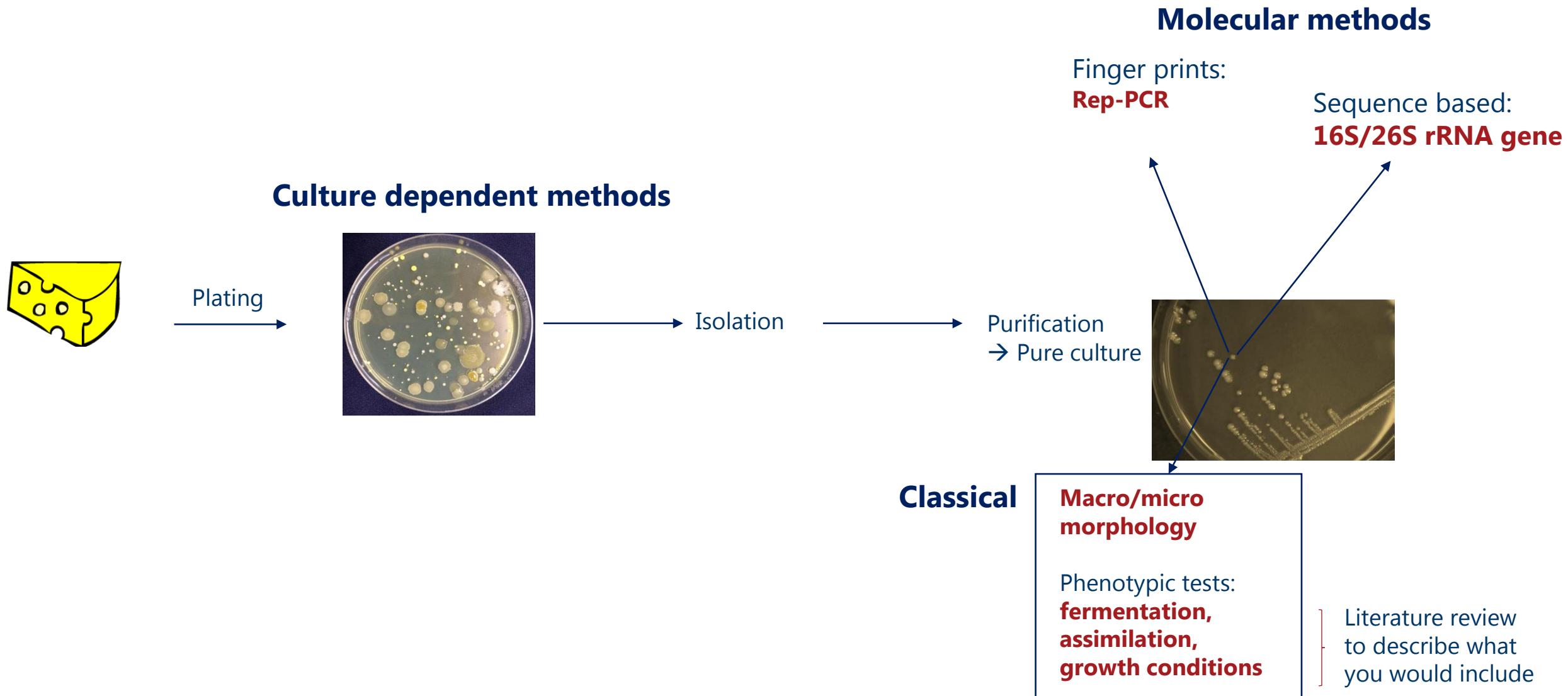
- A high number of **lectures** on topics as identification, taxonomy, microbial ecology, interactions and food fermentation
- **Theoretical cases/quizes** on i) isolation of microorganisms, ii) microbial interactions/cocoa fermentation and iii) fermentation
- **Computer exercises** on primer design, data treatment, sequencing analyses
- **Lab work** on identification of pro- and eukaryotes
- **Lab project** on fermented food (group-wise)



Groups for laboratory work and theoretical exercises

- 20 laboratory groups working with 10 different products/microorganisms, i.e. 1-1, 1-2, 2-1, 2-2,...11-2
 - Bacteria: 5 groups (1-1 to 5-2)
 - Yeasts: 5 groups (6-1 to 10-2)
- The groups are generated randomly. The groups are announced on Absalon under people/project groups.

Identification of microorganisms from food products - an overview



Fermented food products and microorganisms – Lab. work

Group	Product	Organisms	Substrate	Temp	Atmosphere	Time
1-1,1-2	Sourdough (rye)	LAB	mMRS	30°C	Anaerobic	3-5 days
2-1,2-2	Kimchi	LAB	MRS	30°C	Anaerobic	3-5 days
3-1,3-2	Salami	LAB	MRS	30°C	Anaerobic	3-5 days
4-1,4-2	Kefir	LAB	MRS	30°C	Anaerobic	3-5 days
5-1,5-2	Red smear cheese (surface)	Coryneform/ <i>Staphylococcus</i>	TSA + 3% NaCl	25°C	Aerobic	3-5 days
6-1,6-2	Sourdough (rye)	Yeasts	MYGP	25°C	Aerobic	3-5 days
7-1, 7-2	Apple cider	Yeasts	MYGP	25°C	Aerobic	3-5 days
8-1, 8-2	Red smear cheese (surface)	Yeasts	MYGP + 4% NaCl	25°C	Aerobic	3-5 days
9-2, 9-2	White brined cheese	Yeasts	MYGP	25°C	Aerobic	3-5 days
10-1, 10-2	Serrano ham	Yeasts	MYGP	25°C	Aerobic	3-5 days

Presentation of groups work

- Each lab group will generate a power point presentation including current knowledge on the specific food product as well as own results obtained in the lab.
- The ppt presentation should be uploaded in Absalon on 30 October 2024 before 12:00 pm.
- You are allowed to bring print of the slides to the oral exam.

Exam – book the dates!

- Exam is on 5-8/11 2024 (schedule will be planned later – by us).
- External examiner: Søren Lillevang (Arla Foods)
- 10-15 questions will be given to you 2-3 weeks before exam (preferable before week 42).
 - Notice: Exam requirements (curriculum) as uploaded before the course might change slightly during the course.
- The exam
 - Draw one question and answer the question for approximately 8 min.
 - Examination in curriculum and oral presentation of data from the laboratory exercise (5-7 min.)
 - Duration of the examination: max. 20 minutes (including grading)
 - grading primarily for answering the exam question and curriculum. Your understanding of the laboratory exercise will impact the grade as well.
- Scores using the 7-step 12-scale

Safety in the laboratory



Outdoor things/coats and bags

- Overcoats and bags are not tolerated in the laboratories
- There will be space for your bags in the basement – remember to bring a:



Safety instructions

- Available on Absalon
- Please read it and sign (the first day in the lab) that you have read and understood the safety instructions

Safety

- The main aspect: no unintended release of microorganisms to the environment
- Therefore, be sure to collect all supernatants for proper destruction (autoclaving)
- Be careful to disinfect surfaces and hands when you are working and before leaving the laboratory
- It is a requirement that you use a buttoned-up lab coat during your work in the laboratory.
- The lab coat should not be removed from the area of the laboratory.



Handling of microorganisms

- It is strictly forbidden to use mouth pipetting, use micropipettes or pipette helpers
- Avoid aerosols. Use disposable inoculation loops or chilled inoculation needles



Hygiene

- Wash your hands:
 - When you arrive at the laboratory
 - After handling of microorganisms
 - Before you leave the laboratory (breaks)
 - When you finish your work
- It is forbidden to eat, drink and smoke inside the laboratory (chewing gum, too!!)



Disinfections of the working area

- Use 70% ethanol
 - Before the work begins
 - After you have finish your work
 - By spillage



Waste disposal

- Petri dishes in yellow bags
- Plastic tips in a special container on the table (empty the container every day into the yellow bags)
- Object glass in special containers (must be autoclaved)
- Growth media and washing buffer (from centrifugation) are collected in closed containers (autoclaved afterwards)
- Tubes with live cultures are placed into racks before autoclaving



Questions ?



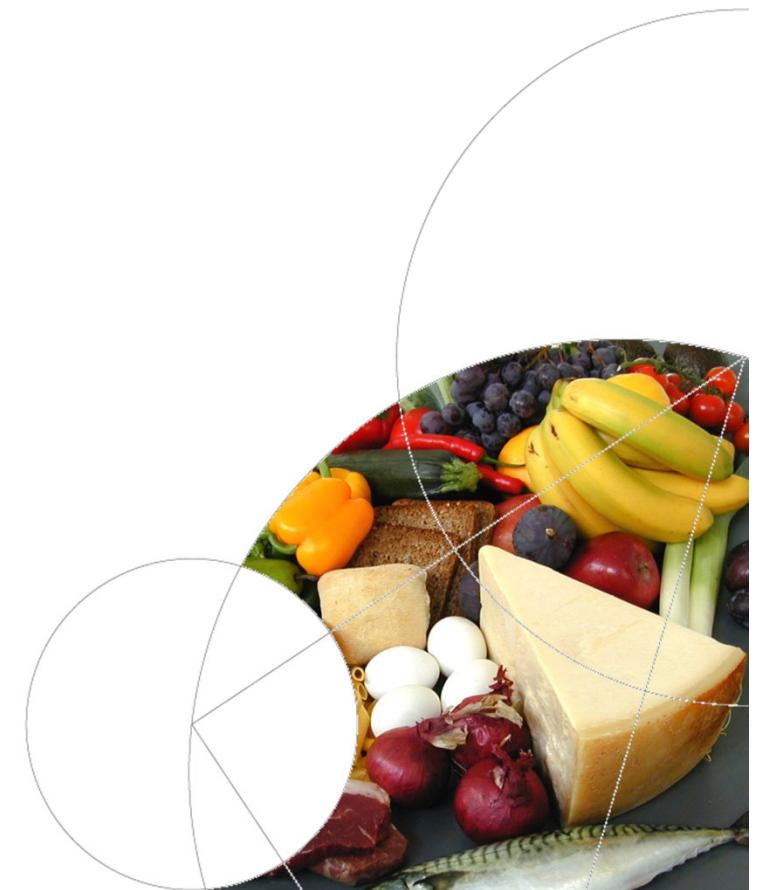
Faculty of Science

DNA, RNA and Protein

Dennis S. Nielsen

dn@food.ku.dk

MFFB 2.9.24
Dias 1



Informational macromolecules – a summary

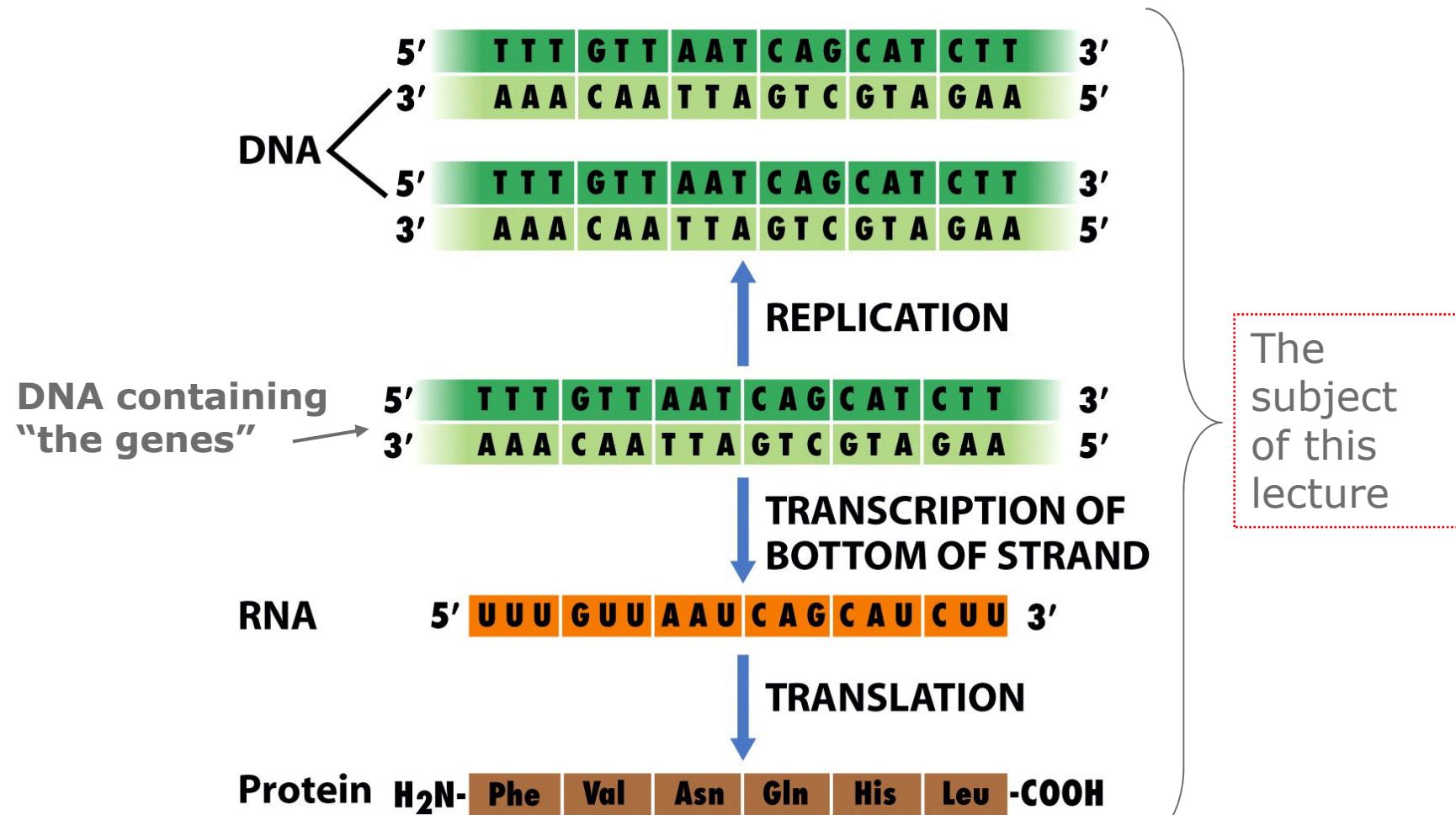


Figure 7-1 Brock Biology of Microorganisms 11/e
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Genetic elements

The chromosome

- Contains all the essential genes (house keeping genes)
- Prokaryotes
 - Usually circular
 - Usually only 1 copy
- Eukaryotes
 - Number of chromosomes vary
 - Linear
 - Located inside nucleus

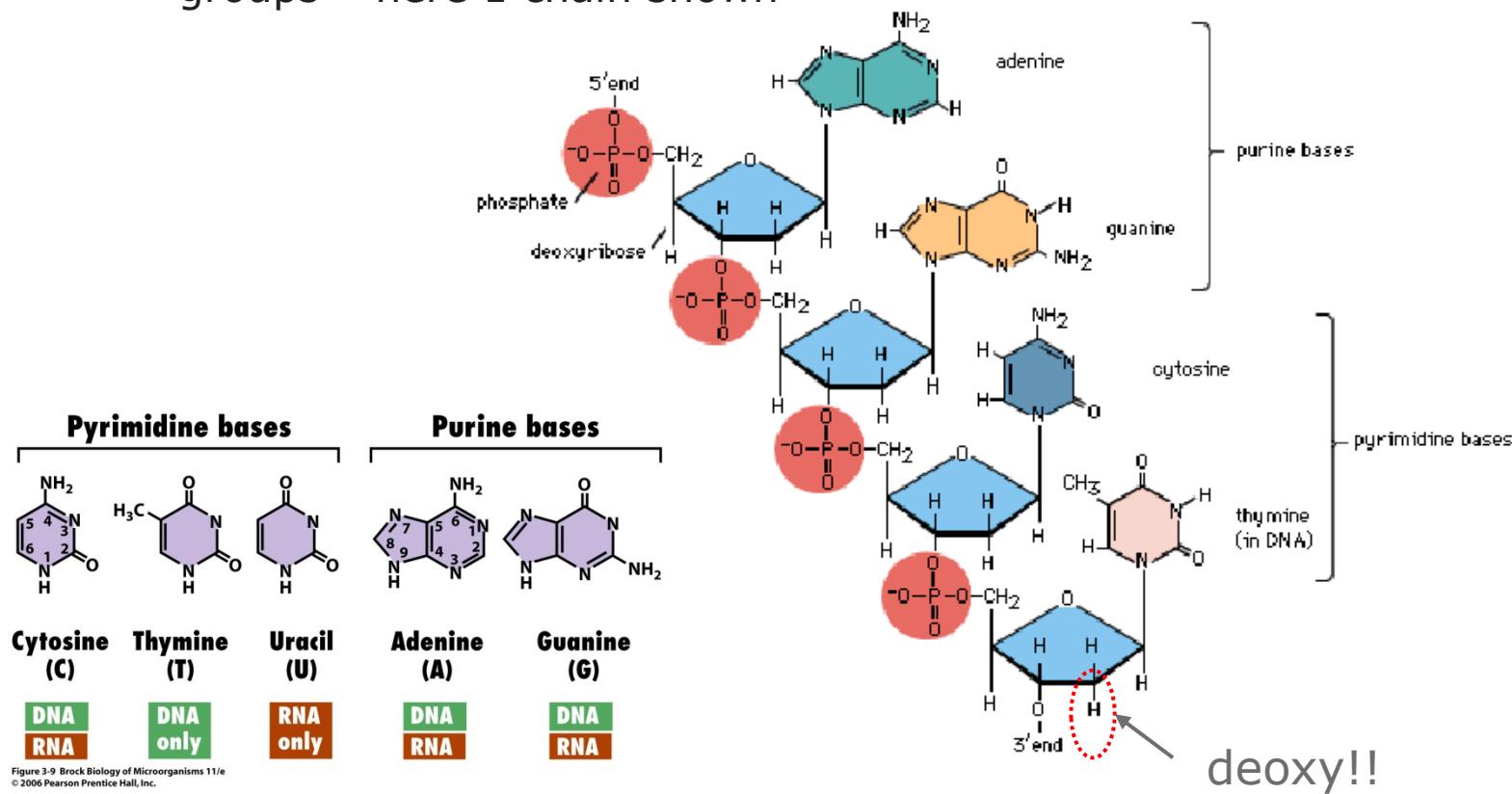
Plasmids

- Relatively short
- Usually circular
- Contains "non-essential" (but often very useful) genes
- Replicates separately (origin of replication)
- 1 or more is common in prokaryotes



DNA structure

- DNA = DeoxyriboNucleic Acid
- Double-helix of 2 chains of complementary nucleotide bases on a backbone of alternating deoxyribose and phosphate groups – here 1 chain shown



DNA structure

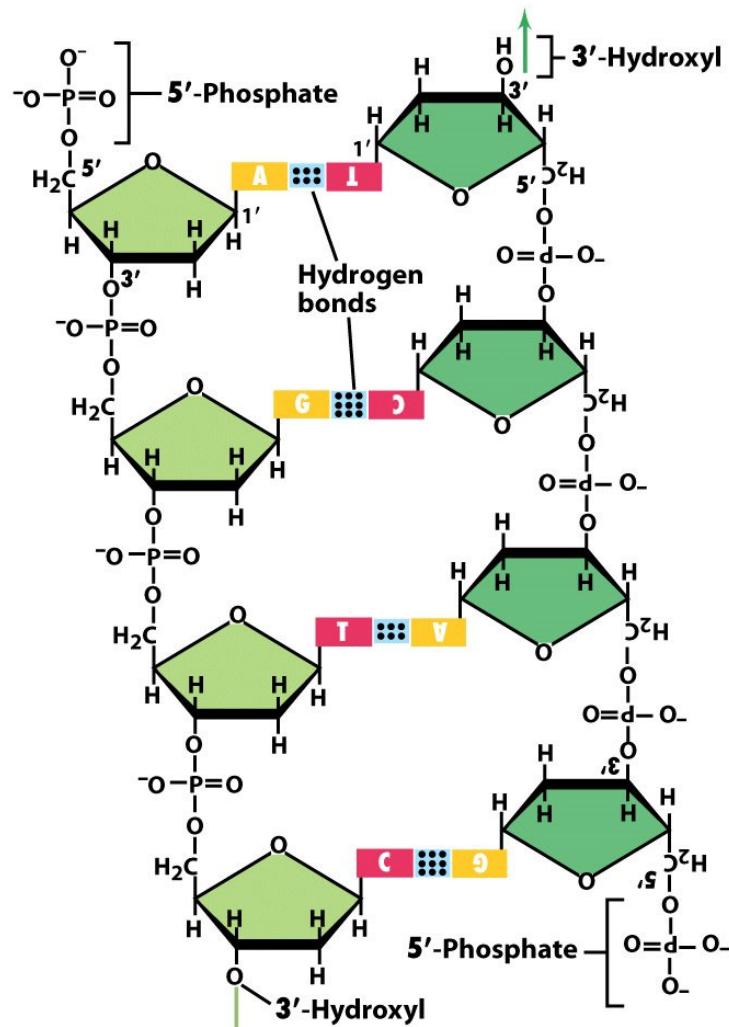


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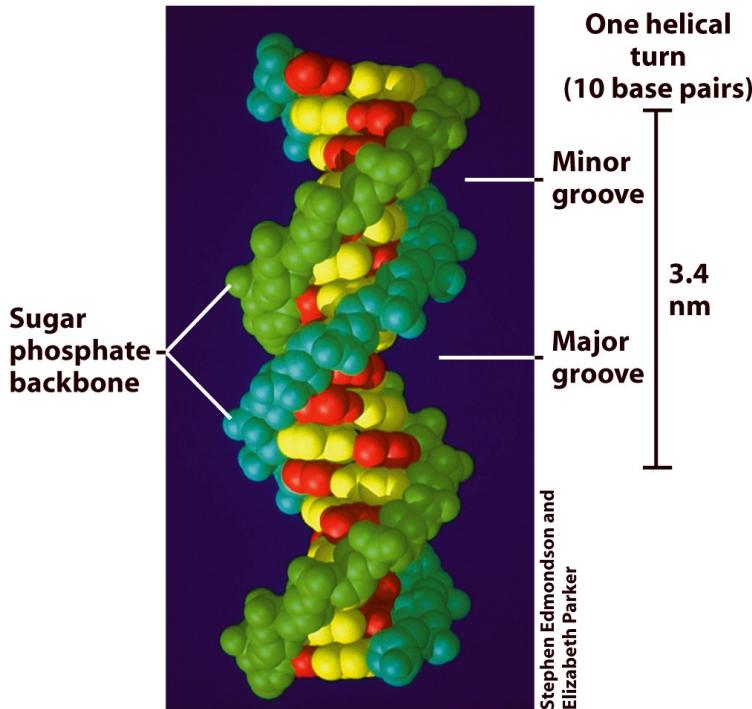
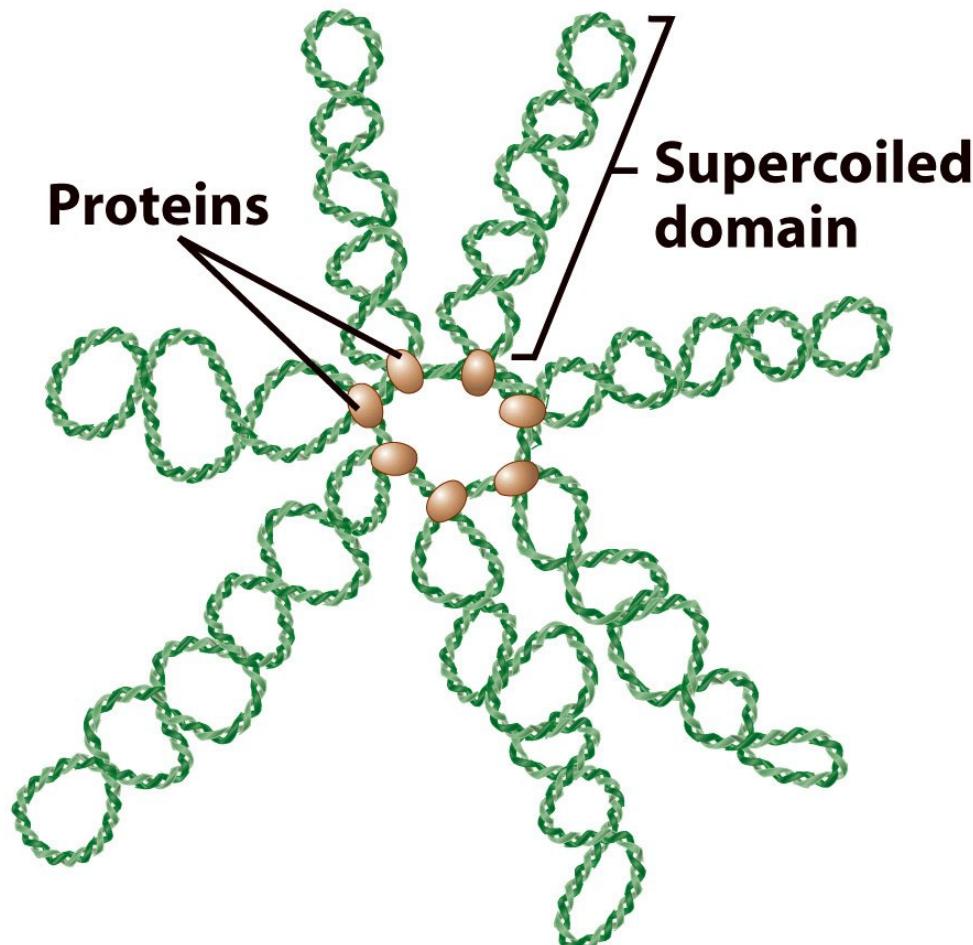


Figure 7-5 Brock Biology of Microorganisms 11/e
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- One base pair: 0.34 nm -> 1000 bp: 0.34 μm long
- An average bacterial genome is 2-5000 kbp (2-5 Mbp)
- That is 0.5-1 mm long...
- How big is a bacterium?



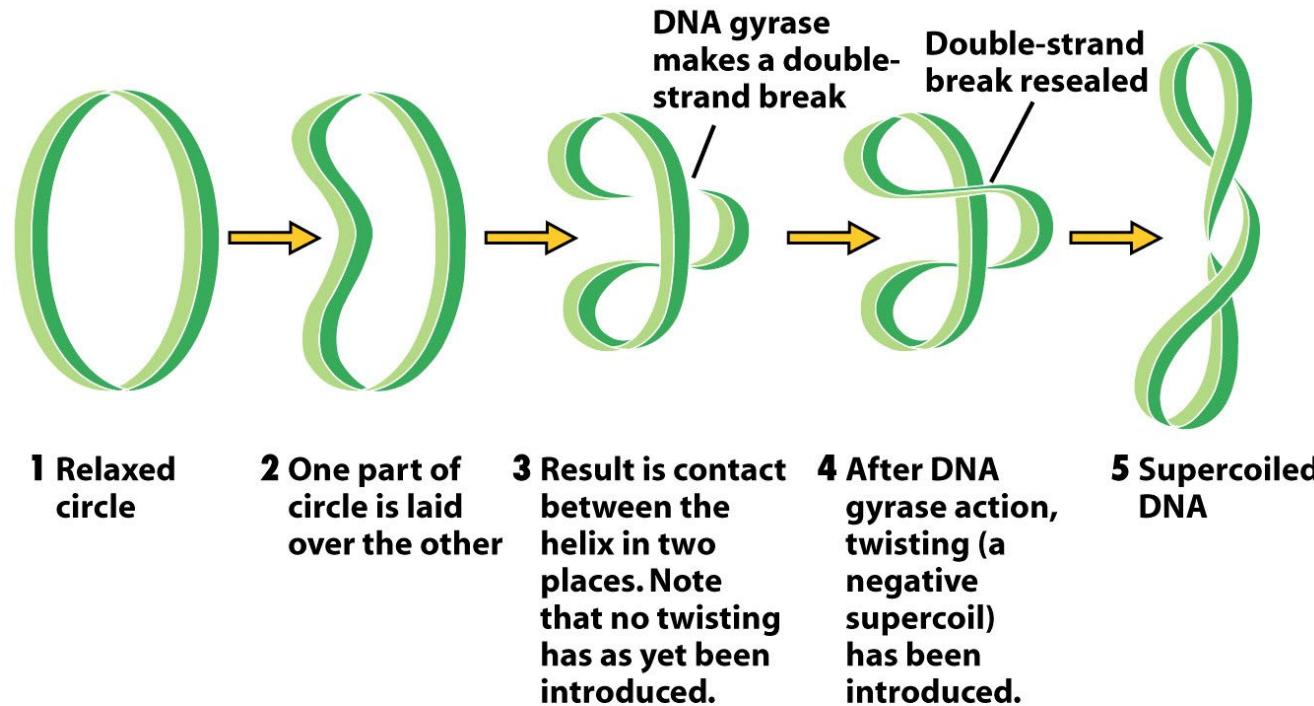
DNA structure – super coiling



Chromosomal DNA with supercoiled domains



DNA structure – supercoiling



In *Bacteria*:

- Introducing supercoils: *Topoisomerase II* (a DNA gyrase, target of many antibiotics)
- Removing supercoils: *Topoisomerase I*



DNA replication

- DNA replication = copying of DNA
- The essential enzymes here are *DNA polymerases*
 - Catalyses the addition of deoxyribonucleoside triphosphate at the 3' end of the chain
 - DNA is ALWAYS(!!!) synthesised in the 5'→3' direction

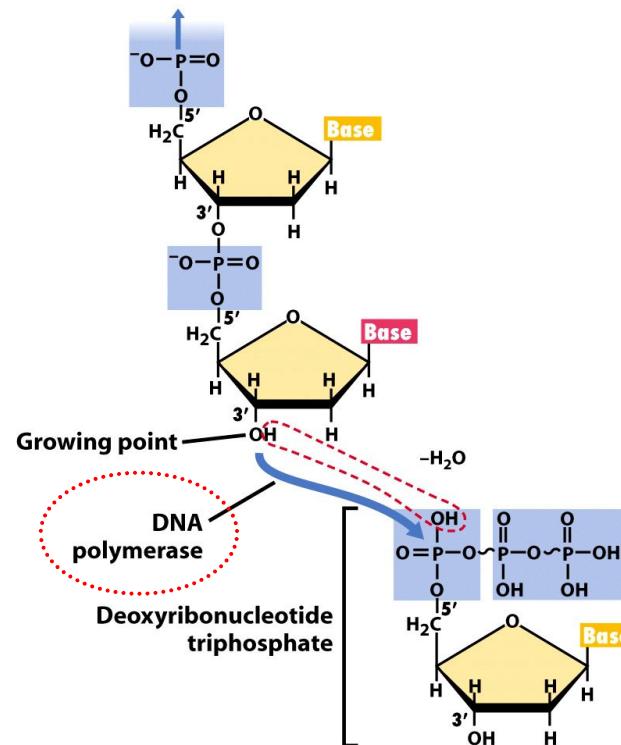


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DNA replication

- All known DNA polymerases can only add a nucleotide to a pre-existing 3'-group
- A primer is needed to start the replication
- When replication starts the enzyme *primase* synthesises a short RNA-strand complementary to the template DNA
- At the 3' end of this primer there is free OH-group to which DNA polymerase can add the first deoxyribonucleotide



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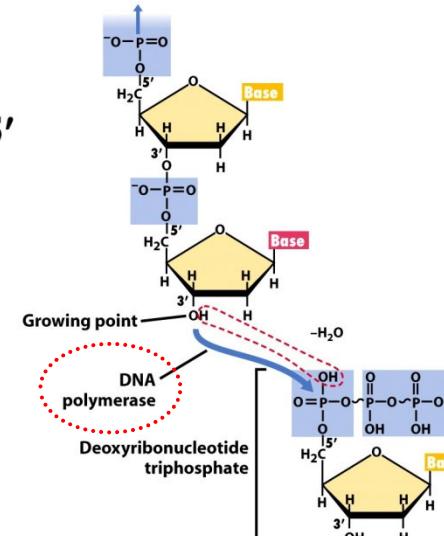


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DNA replication

- Both strands are copied during replication
 - Semi conservative
- The “DNA replicating complex” moves along the double stranded molecule
- Meaning that one strand will be copied in $5' \rightarrow 3'$ direction and one strand seemingly in the $3' \rightarrow 5'$ direction
- **How is this possible??**

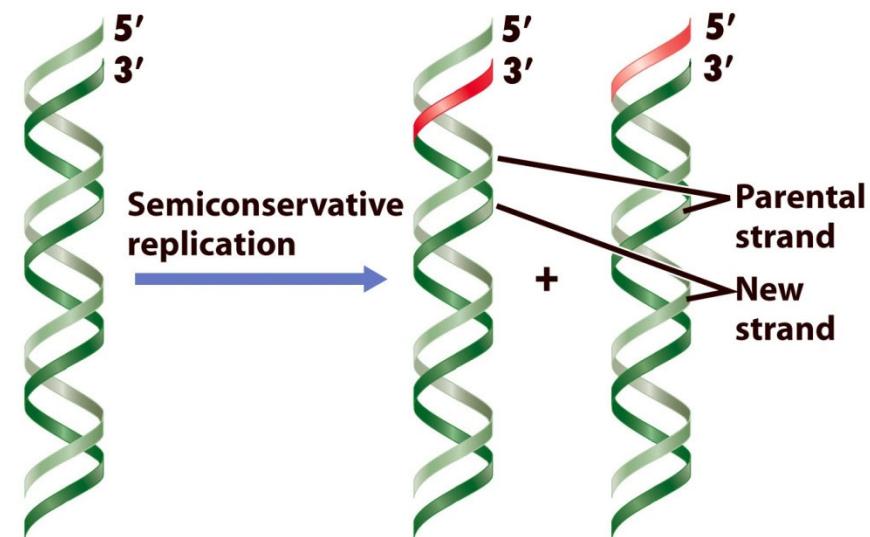
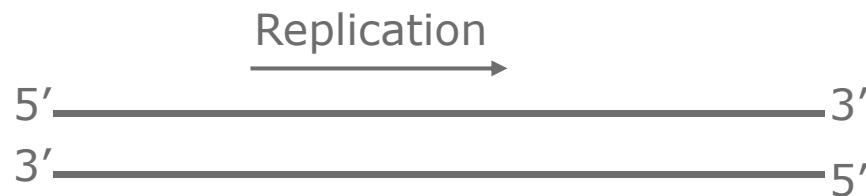


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The DNA replication fork

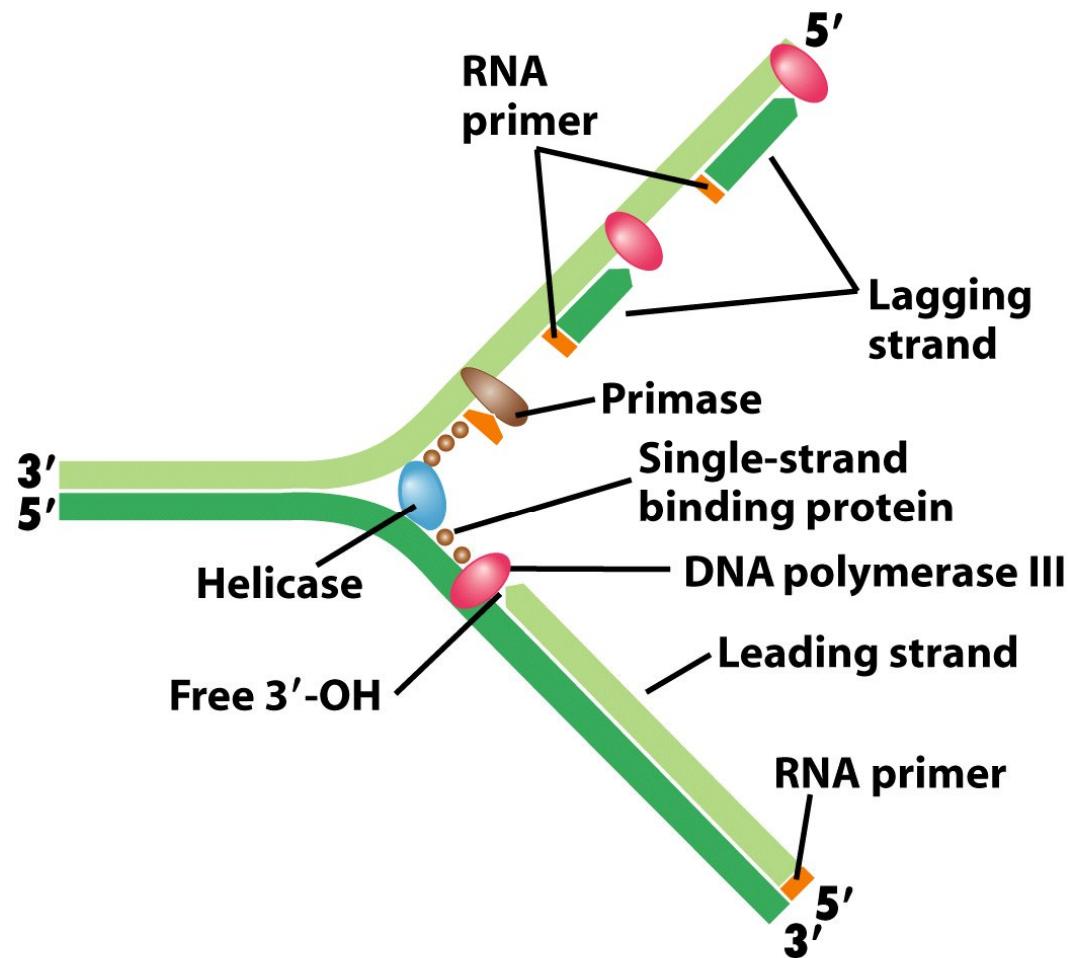


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A more detailed look on the lagging strand

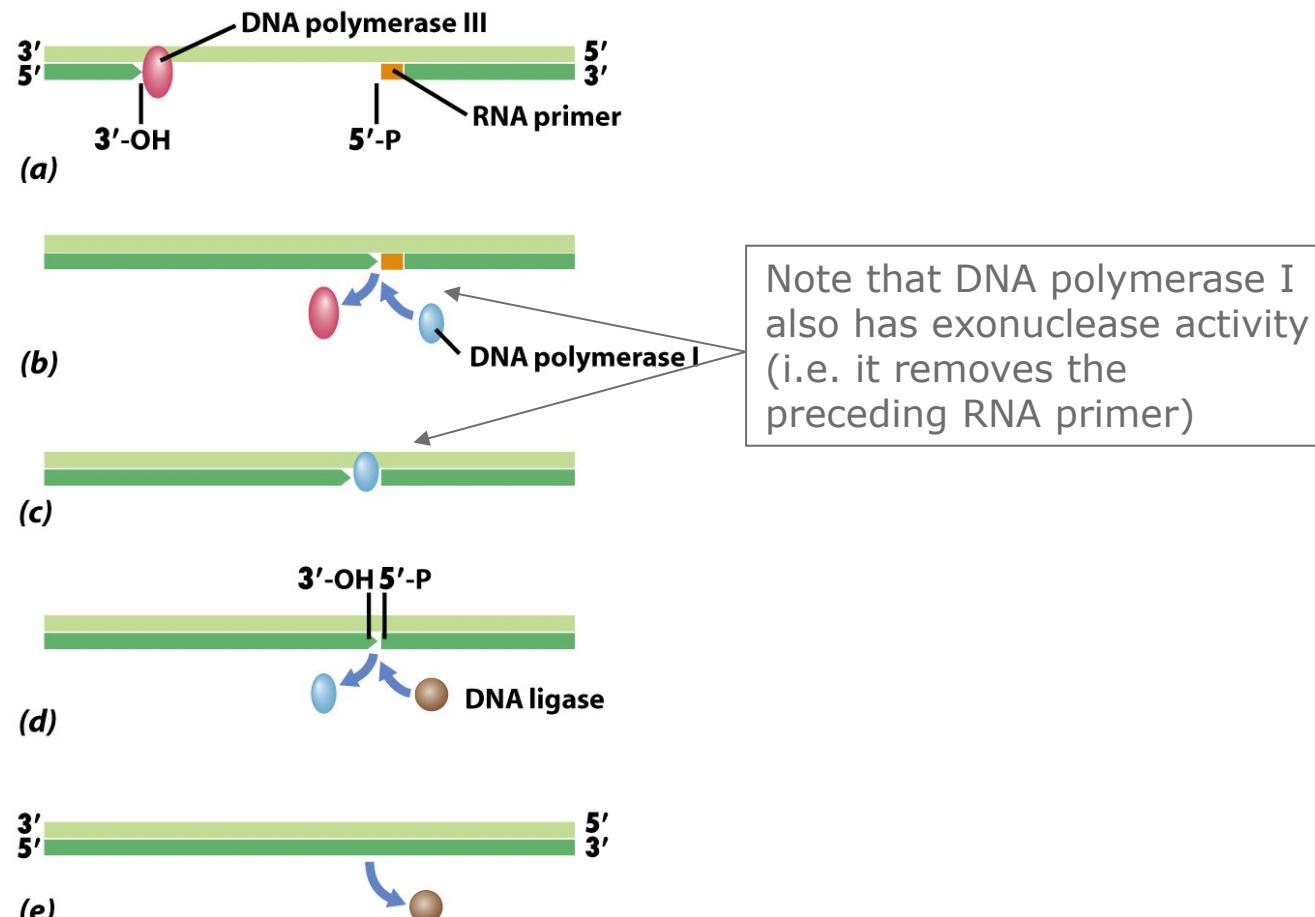


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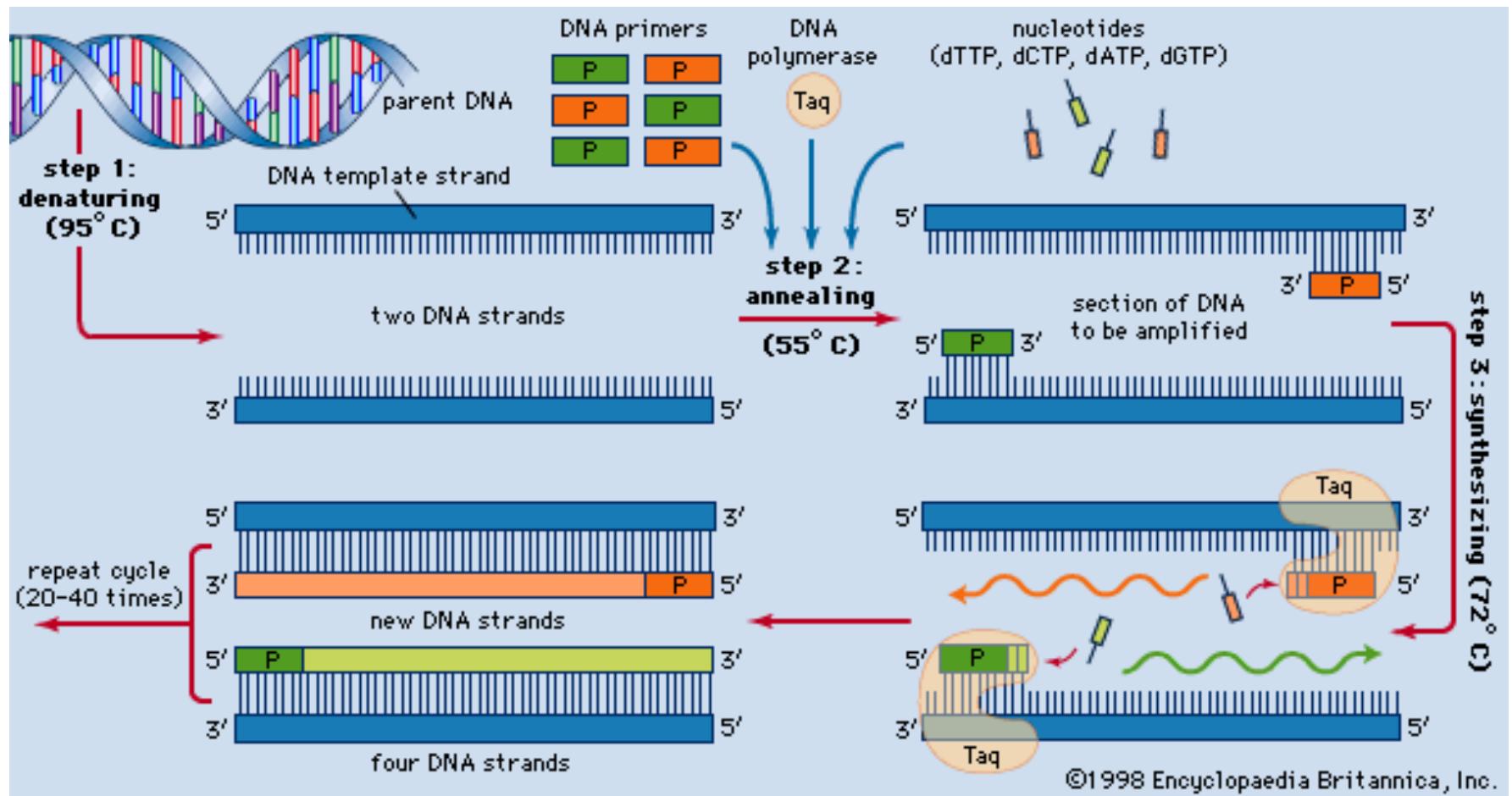


Manipulating DNA – The polymerase chain reaction

- PCR = Polymerase Chain Reaction
- Used to copy fragments of DNA in a test tube
- The basis for many molecular biology methods
- DNA is copied using a chemically synthesised primer and a polymerase (usually DNA polymerase III)
- The process involves high temperatures (95 ° C) and the reaction was not really efficient until the isolation (and cloning) of a polymerase from the thermophilic bacterium *Thermus aquaticus* (the *Taq* polymerase)
- PCR is a thermocycling process where the temperature is raised and lowered in a sequential manner using a “Thermocycler”



Polymerase chain reaction



Let's animate that!

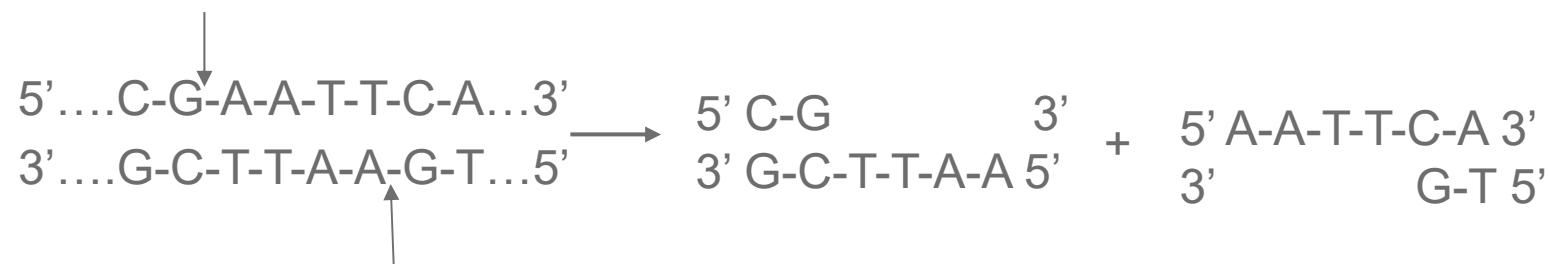
<https://www.youtube.com/watch?v=2KoLnIwoZKU>

This one <https://www.youtube.com/watch?v=iQsu3Kz9NYo> is longer and a bit more thorough (and has dramatic music playing)



Restriction enzymes

- Restriction enzyme: An enzyme that recognises and makes double-stranded breaks at specific DNA sequences
- Defends the cell against foreign (virus) DNA
- E.g. *EcoRI* (restriction enzyme purified from *Escherichia coli*):



- In molecular biology: Used to cut DNA at specific, well defined sites/sequences



A little excersise:

PCR & restriction enzymes

You have 10 minutes. Work 2 and 2.



Ribonucleic acid (RNA)

- Three key differences between Ribonucleic Acid (RNA) and DNA
 - The sugar backbone consists of ribose, not deoxyribose
 - Contains the base uracil instead of thymine
 - Single-stranded (except in a few viruses)
- Four major types of RNA are known
 - mRNA = messenger RNA
 - tRNA = transfer RNA
 - rRNA = ribosomal RNA
 - miRNA = microRNA
- RNA functions at 2 (sometimes 3) levels in the cell
 - Genetic: Carries genetic information from DNA via mRNA
 - Functional: tRNA and rRNA are macromolecules in their own right with important functions in the ribosomes and during protein synthesis
 - miRNA: RNA silencing and post transcriptional regulation of gene expression



Transcription of DNA – RNA synthesis

- The chemistry of RNA synthesis is much like the synthesis of DNA, but (of course) with a few differences
- Transcription of DNA to RNA is carried out by RNA polymerase
 - Prokaryotes: Single polymerase
 - Eukaryotes: RNA polymerase I (rRNA), II (mRNA) and III (tRNA)
 - $5' \rightarrow 3'$ direction
 - Can initiate synthesis *de novo* – i.e. no need for primer
 - RNA polymerase consist of 4 sub-units [β , β' , α (2 copies) and σ]
 - Forms 2 units weakly bound to each other: The core enzyme ($\alpha_2\beta\beta'$) and σ (the "sigma" factor)
- In prokaryotes: The sigma factor recognise "where to start" = the promoter region
- In Eukaryotes: Same-same, but different names... Promoter often the so-called TATA-box.
- The core enzyme carries out the actual elongation
- Transcription stops at so-called transcription terminators



Transcription (bacteria)

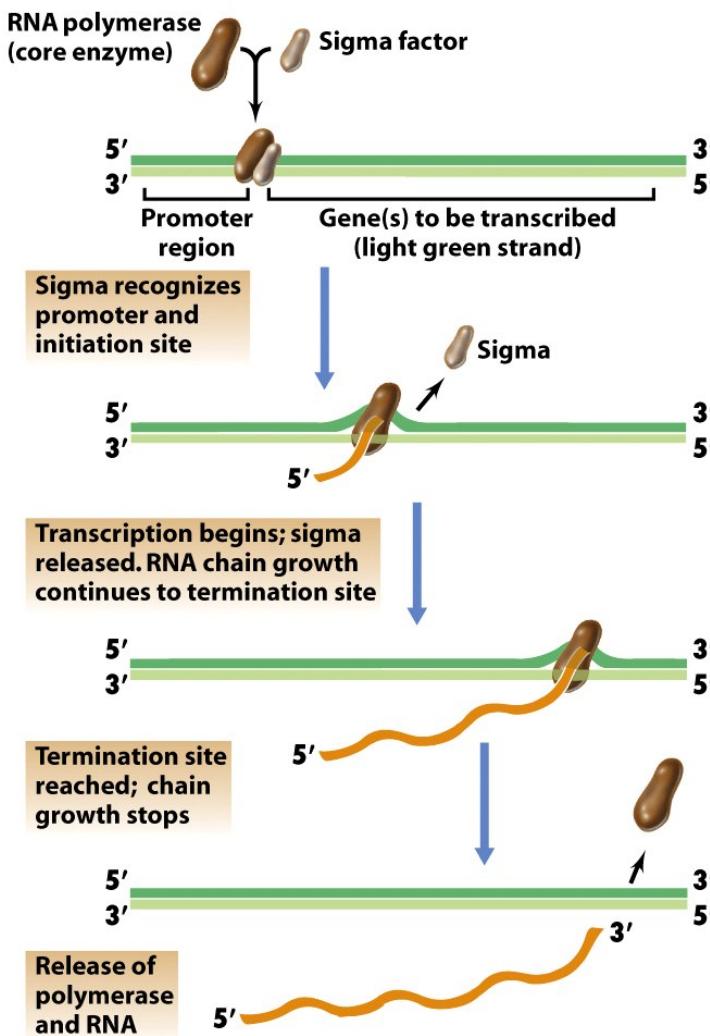


Figure 7-29a Brock Biology of Microorganisms 11/e
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Promoter regions

- **Promoter regions** are “correct places to start transcription” recognised by sigma (σ) (transcription) factors
 - Most organisms contain several σ factors
 - Normally 1 “general” σ factor takes care of transcribing the majority of genes, and a number of specialised σ factors transcribing rarely needed genes
 - Note the **consensus sequence** in the figure
 - Strong promoters have sequences very similar to the consensus sequence

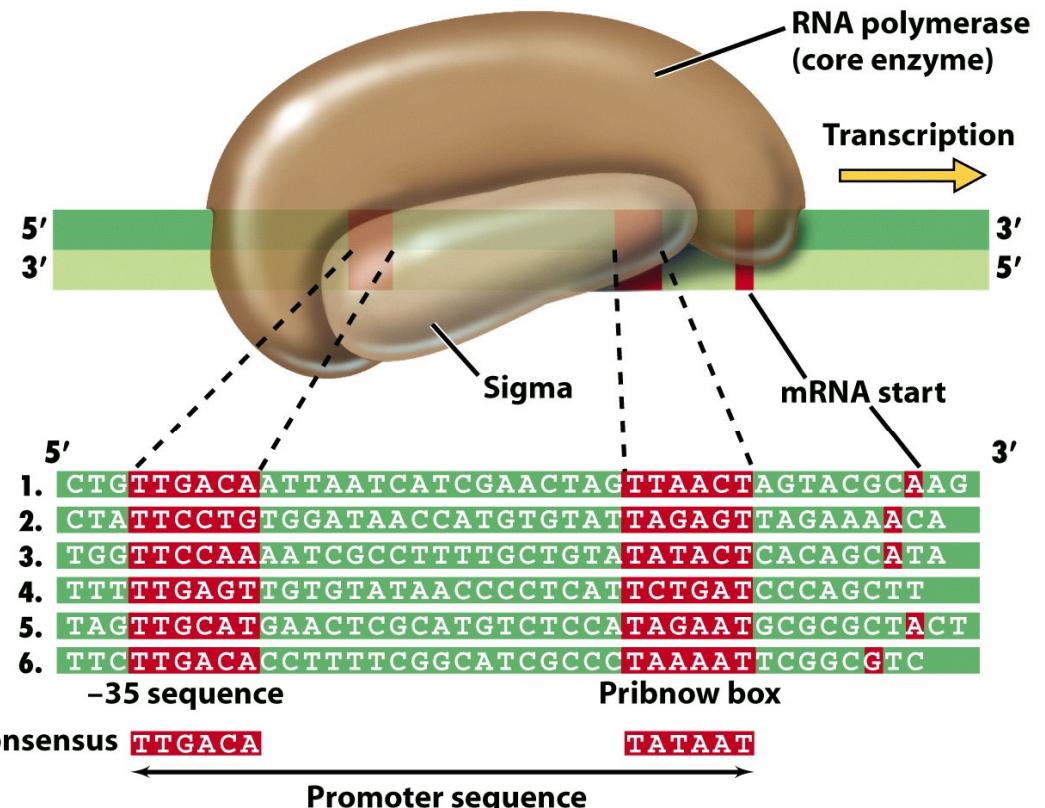
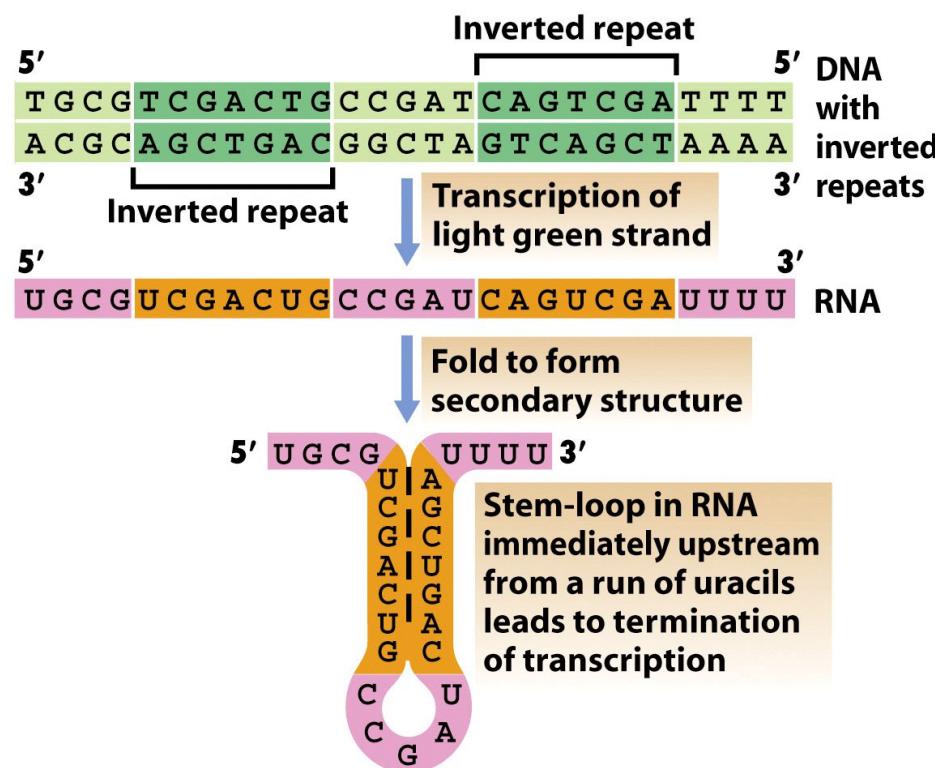


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Transcription terminators

- Or “where to stop”?
- A common terminator is “an inverted repeat”
- Other types are protein dependent terminators – e.g. the *Rho*-system in *E. coli*, *B. subtilis* and other *Bacteria*



The ribosomes

- Following transcription mRNA is translated into proteins in the ribosomes
- The ribosome consists of ~ 54 (prokaryote)/80 (eukaryote) proteins and 3 (prokaryote)- 4 (eukaryote) ribosomal RNA's (rRNA)
 - 5S (prokaryotes) / 5S & 5.8S (eukaryotes)
 - 16S / 18S
 - 23S / 26S or 28S
- Organised into 2 subunits (large and small)
- The rRNA's are essential for
 - recognition/correct binding of mRNA in ribosomes
 - maintaining the right structure of the ribosomes
- Furthermore transfer RNA (tRNA) is essential in protein synthesis (more to follow)
- rRNA and tRNA both fold into advanced secondary structures
- This is protecting them against degradation by ribonucleases given them a long half life (contrary to mRNA)



Secondary structure of 16S rRNA

- 16S rRNA (and hence also the 16S rRNA gene) contains a number of **variable** (9) and **conserved** regions
- The variable regions are highly variable in sequence between even closely related species
- The conserved regions are on the other hand conserved between even distantly related organisms

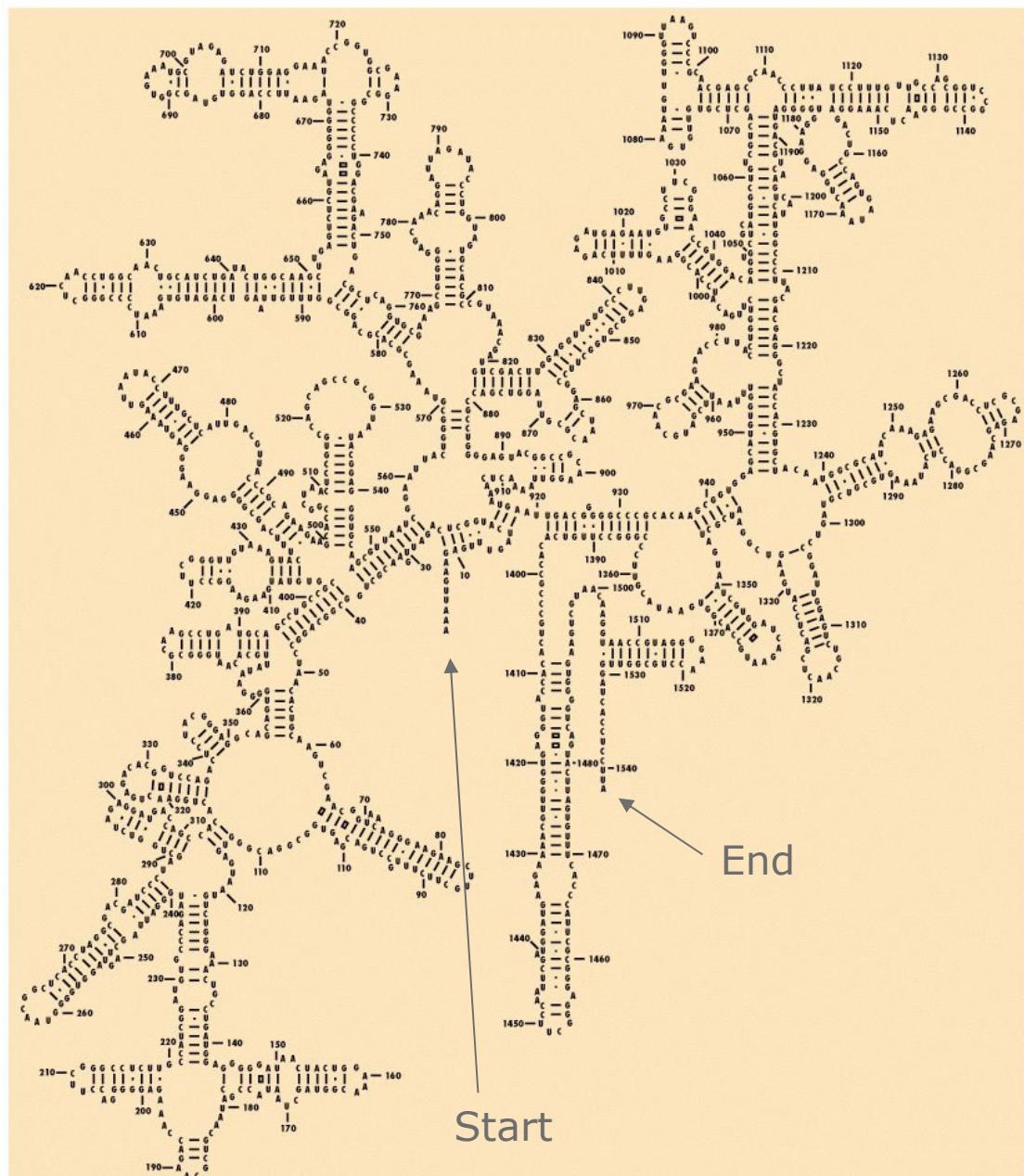
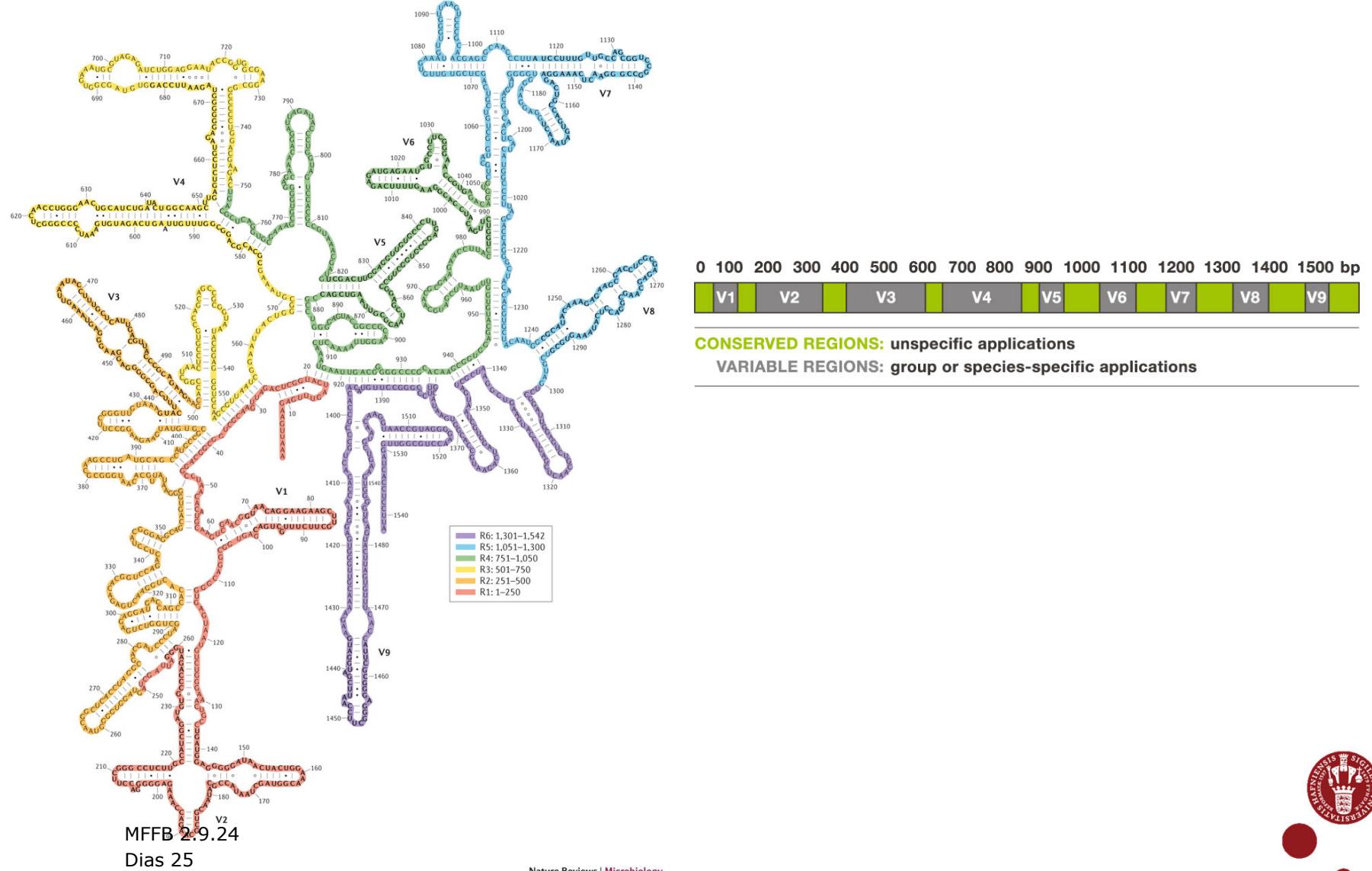


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16S rRNA gene structure (and primer design)



Transcription of rRNA and tRNA

- Genes encoding related enzymes are often organised in operons where they cluster together on the chromosome (in prokaryotes)
- Expression often controlled by an operator (promoter)
- Also rRNA and tRNA is organised in operons (the rRNA operon)

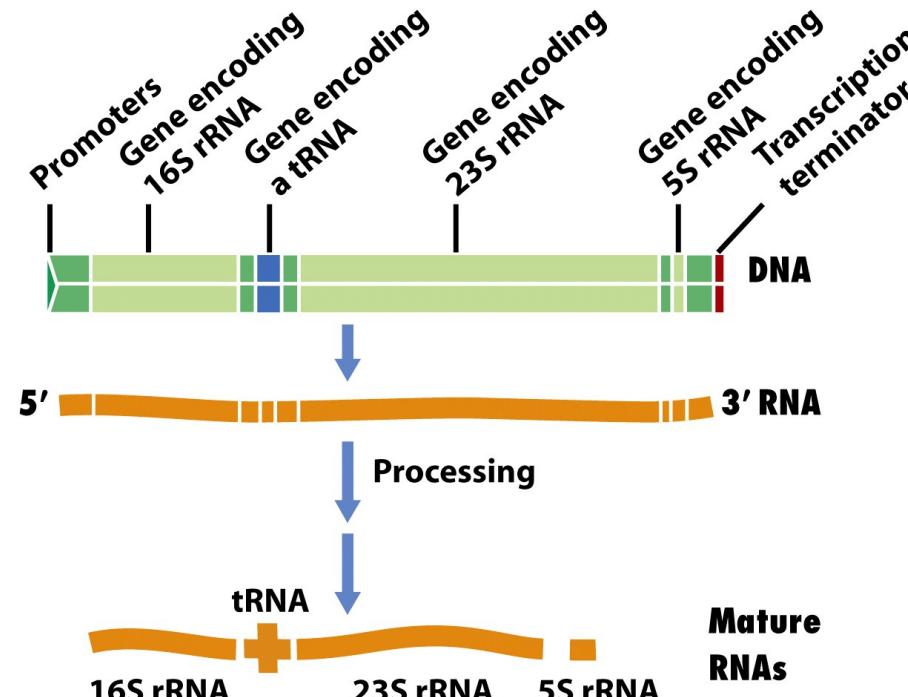


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Translation

- Translation of mRNA into protein is the last step in the genetic information transfer from DNA over RNA to protein
- The basis is “the genetic code” where a triplet of 3 bases (also called a codon) translates into a specific amino acid (AA)
- There are 64 possible codons (4^3) of mRNA
- Several AA's are encoded by several different (but related) codons
 - 64 different codons, but only 22 different AA
 - I.e. knowing the AA at a specific position does not necessarily mean you also know the codon
- In addition there are specific “start” and “stop” codons



Codons

Table 7.5 The genetic code as expressed by triplet base sequences of mRNA^a

Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
UUU	Phenylalanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine
UUC	Phenylalanine	UCC	Serine	UAC	Tyrosine	UGC	Cysteine
UUA	Leucine	UCA	Serine	UAA	None (stop signal)	UGA	None (stop signal)
UUG	Leucine	UCG	Serine	UAG	None (stop signal)	UGG	Tryptophan
CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine
CUC	Leucine	CCC	Proline	CAC	Histidine	CGC	Arginine
CUA	Leucine	CCA	Proline	CAA	Glutamine	CGA	Arginine
CUG	Leucine	CCG	Proline	CAG	Glutamine	CGG	Arginine
AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine
AUC	Isoleucine	ACC	Threonine	AAC	Asparagine	AGC	Serine
AUA	Isoleucine	ACA	Threonine	AAA	Lysine	AGA	Arginine
AUG (start) ^b	Methionine	ACG	Threonine	AAG	Lysine	AGG	Arginine
GUU	Valine	GCU	Alanine	GAU	Aspartic acid	GGU	Glycine
GUC	Valine	GCC	Alanine	GAC	Aspartic acid	GGC	Glycine
GUA	Valine	GCA	Alanine	GAA	Glutamic acid	GGA	Glycine
GUG	Valine	GCG	Alanine	GAG	Glutamic acid	GGG	Glycine

^aThe boxes of codons are colored according to the scheme: ■ ionizable: acidic, □ ionizable: basic, ■ nonionizable polar, and ■■■ nonpolar (Figure 3.12). The nucleotide on the left is at the 5'-end of the triplet. Note that certain stop (nonsense) codons do not always function as such in certain organisms (see text and the Microbial Sidebar, Unconventional Amino Acids).

^bAUG encodes N-formylmethionine at the beginning of mRNAs of *Bacteria*.

Table 7-5 Brock Biology of Microorganisms 11/e

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Start and stop codons

- It is essential to start translation in the right position, otherwise "frameshifts" leading to (most probably) not functioning proteins will happen
- The *start codon* (5' AUG 3') is (in prokaryotes) identified by the ribosomes by the aid of an up-stream (~ 8 bp) so-called Shine-Dalgarno (SD) sequence (consensus sequence: 5' AGGAGG 3', in *E. coli* AGGAGGU) complementary to a sequence located on the 3' end on the 16S rRNA (= the anti SD sequence)
- In prokaryotes the start codon almost always encodes N-formylmethionine. In eukaryotes: methionine
 - Alternative start codons: GUG and UUG
- A few codons does not encode an AA (UAA, UAG, UGA). These "non-sense" codons acts as *stop codons* terminating translation
- A start codon followed by a number of codons and finally a stop codon is called an *open reading frame* (ORF)



Functioning and structure of tRNA

- tRNA are short (73-93 nucleotides long) molecules with a special secondary structure
- A key feature is the *anti-codon* that recognises the codon on the mRNA
- tRNA's are specific for specific AA's (determined by their anti-codon)
- Transfer the AA to the growing protein

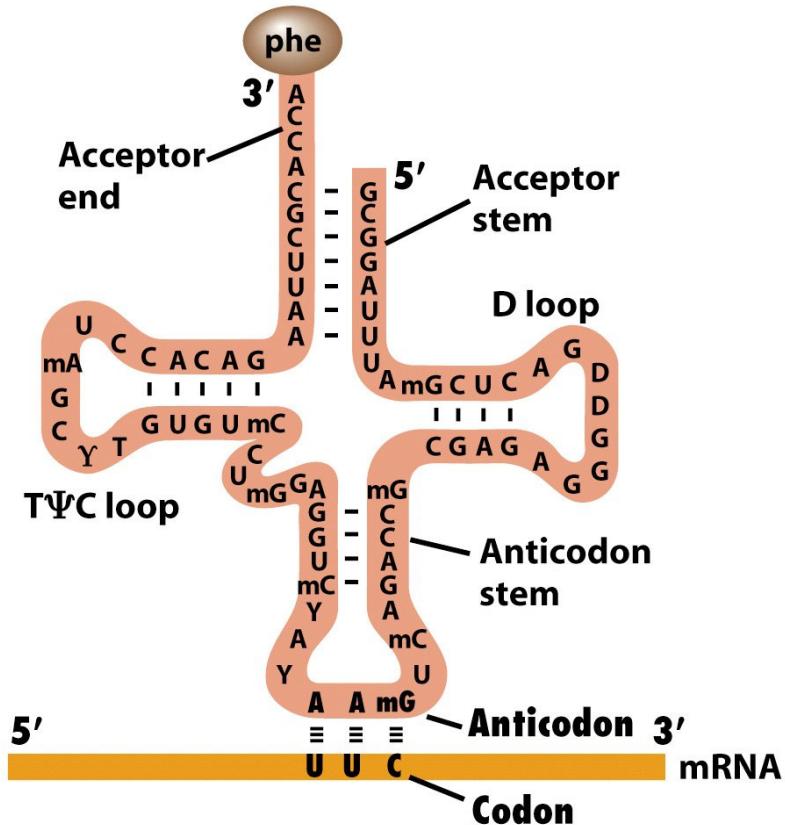
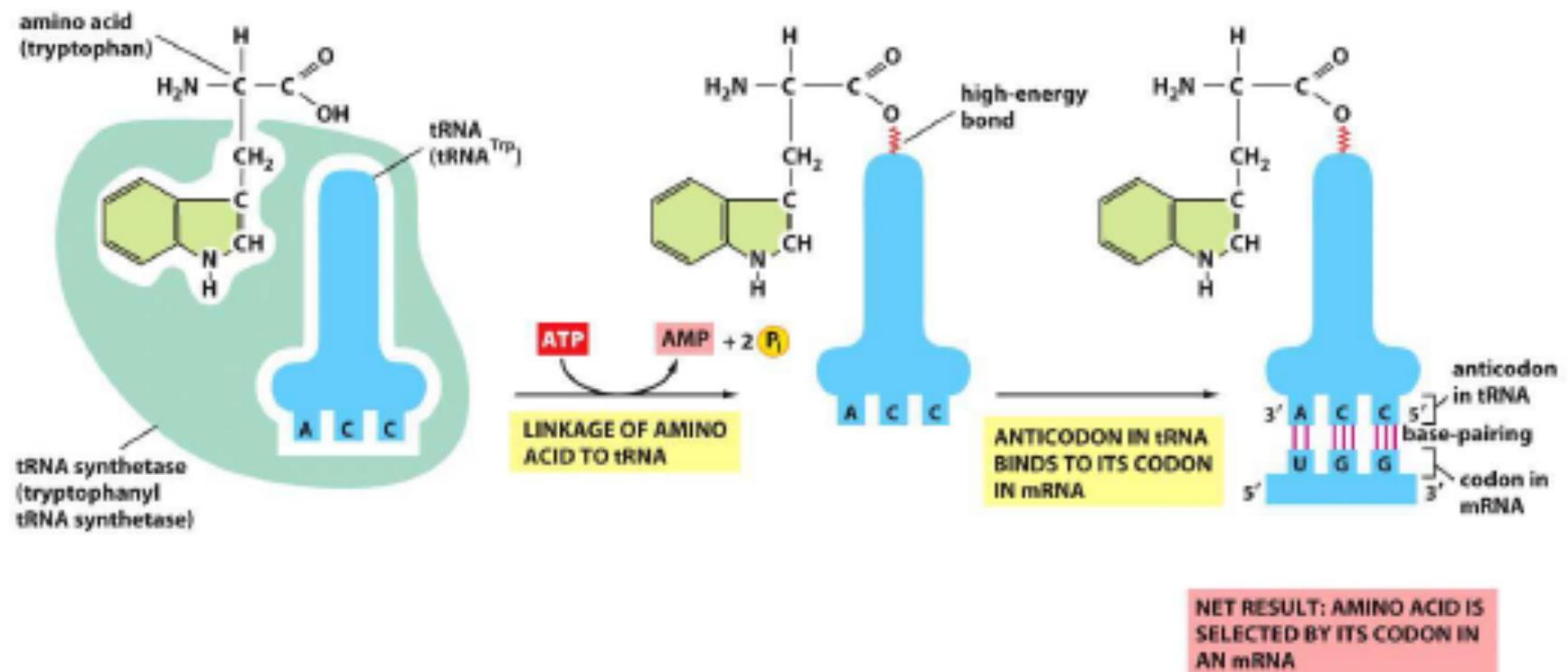
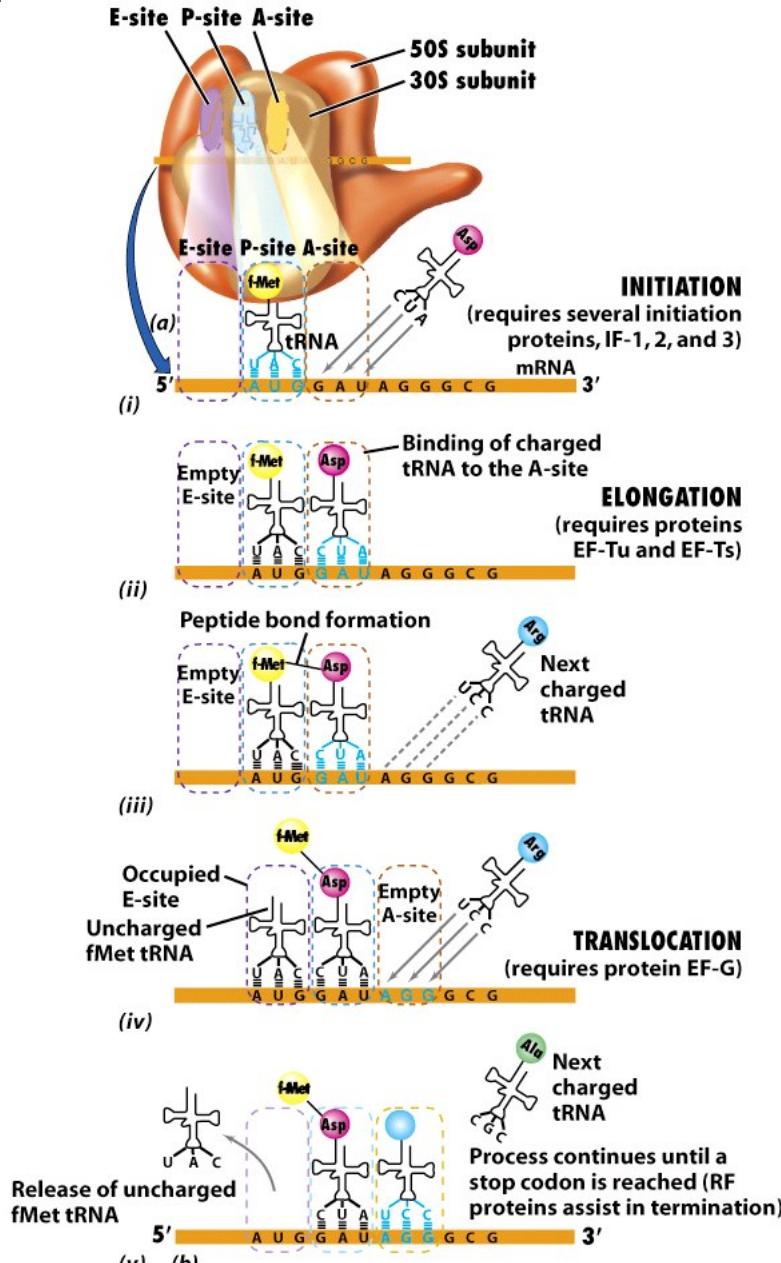


Figure 7-36a Brock Biology of Microorganisms 11/e
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tRNA in action (1)





FigMFB-28B24 Biology of Microorganisms 11/e
© 2006 Pearson Prentice Hall, Inc.

tRNA in action (2)/Protein synthesis

- Two sub-units (the large and the small sub-unit) form the ribosomes
 - A-site = Acceptor site where the tRNA first attaches
 - P-site = Peptide site where the new peptide bond is formed
 - E-site = Exit site where the "empty" tRNA is released
- EF = elongation factors
- RF = release factors
- Translocation = moving 1 codon down the mRNA
- Note that several ribosomes can translate the same mRNA simultaneously forming a *polysome*



Folding and secreting of proteins

- Following synthesis the protein must fold into the active form
 - Often spontaneously
 - Or aided by molecular *chaperones* folding and/or assembling the proteins in the active form and/or complex
 - Chaperones has re-folding activity helping the cell to cope with various stresses
 - Chaperones are thus often referred to as Heat Shock Proteins (HSP)
- Many proteins are active in the cytoplasmic membrane or perhaps even outside the cells
 - Proteins that must be transported over membranes are often synthesised with a signal sequence telling that this protein is to be transported to a given location in (or outside) the cell



Confused??

No need to worry

- Read e.g. "Brock Biology of Microorganisms" 14th edition
 - Chapter:4 "Molecular microbiology" (pp. 149-151 suppl. reading)
 - Chapter 6: "Microbial genomics" pp. 208-214.
- Show up for theoretical/computer exercise Wednesday the 11th





Food microbiology Fermentation (and fermented foods)

Henrik Siegumfeldt



"positive" microbiology vs "negative" microbiology

For most consumers, microbiology and microorganisms in food is associated with negative connotations

There are two issues that are normally considered as "negative" microbiology

1. Food safety: Examples are outbreak of food poisoning caused by bacteria (e.g. *Salmonella*, *Campylobacter*, "the burger bug" etc.)
1. Food quality: Examples are rotting meat and vegetables, bread and jam covered with mould, or sour beer

However, most of the consumers "forget" that on a daily basis, they consume food (both staple food and delicacies), that have been part of microbial growth/microbial conversion. This is what we normally call **fermented foods**



What is fermentation

1. An anaerobic conversion of a substrate to a product using substrate level phosphorylation. - This is a biochemical definition, and also covers e.g. the build-up of lactate in muscles during an anaerobic workout.



2. A microbial conversion in a food (or feed), that causes enhanced shelf life, improved flavour, and or increased nutritional value. – This is a more generic definition, that also includes biochemical changes that does not comply with definition 1.

Examples are “fermentations” with moulds (that are usually aerobic) and vinegar



Fermented products by raw material

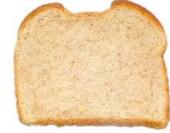
Fermented dairy products

- Cultured milk, butter, cheese ...



Fermented cereal products

- Yeast-leavened bread, sourdough bread, beer, indigenous fermented cereal gruels, (soy sauce)...



Fermented meat products

- Fermented sausages, dried hams ...



Other fermented products

- Wine, fermented vegetables, sauerkraut, mead, kombucha ...



Fermented products by type or end product

Acid fermented products

- Typically performed by lactic acid bacteria

Alcoholic fermentations

- Typically performed by yeast

Alkaline fermentations

- Typically performed by *Bacillus* spp.

Vinegar (acetic acid) production

- Typically performed by *Acetobacter/Gluconobacter*

Mould converted products

- Typically performed by *Penicillium* and *Aspergillus* spp.



Fermented products by technology

Spontaneous fermentation vs. defined starter cultures

Single culture fermentations

- E.g. beer

Co-culture fermentations

- E.g. yoghurt

Successive fermentations

- Cocoa, sauerkraut, mould ripened cheeses ...

“Fermentations” with enzymes

- E.g. Soy sauce



Temperature is important

Most fermentations are performed at “ambient” temperature, which means that the fermentations are performed at the “natural” ambient temperature in the area of the world where the process was developed.

However, some fermentations are colder, e.g. Lager (beer) fermentation (8-14 ° C)
And some are warmer, e.g. Yoghurt (39-42 ° C)

When you have a mixed culture fermentation, the temperature can affect the ratio between microorganisms. This can alter the sensory quality, e.g. Kombucha becomes more acidic (vinegar) when the temperature increases.

The size of the fermenter influences temperature control, as large volumes respond slowly to changes in the external temperature.



Presence of oxygen is also important

Most fermentations are anaerobic, and the presence of oxygen will therefore favour the growth of unwanted microorganisms.

However, two types of “fermentation” requires oxygen, namely fermentations involving acetic acid production (e.g. Kombucha), and any process where the growth of filamentous fungi (moulds) is required.

As mentioned under temperature, the size of the container should also be considered, as it can be difficult to aerate large containers.



Typical addition of chemical compounds to fermented products

The most predominant chemical is table salt (NaCl), which has the ability to lower water activity, and thereby prevent the growth of unwanted microorganisms.

Sugar (sucrose, glucose, fructose) can also be added with the purpose of lowering water activity, but it can also be added as a fermentable substrate (e.g. certain beers, kombucha)

In certain types of fermented products, addition of spices (e.g. garlic) can also act as a source of fermentable carbohydrates.



Typical organisms involved in fermentation – I - "safe" organisms

Bacteria:

Typically Lactic acid bacteria, that convert pyruvate to either lactate (homofermentative) or lactate and CO₂ and ethanol (heterofermentative).

These bacteria have a GRAS status (Generally Regarded As Safe), which means that they usually do not cause food SAFETY problems. However they may cause food SPOILAGE problems (sour odour in many RTE (Ready to Eat) products)

Yeast: typically *Saccharomyces cerevisiae*, that convert pyruvate to ethanol and CO₂. This is utilised in products primarily aimed at obtaining ethanol (e.g. wine), products where CO₂ is most desired (e.g. yeast-leavened bread), and products that contain both end products (e.g. beer). *Saccharomyces* is also GRAS



Typical organisms involved in fermentation –II- “risky” organisms

Mould fungi: Typically from few genera, often *Aspergillus*, and *Penicillium*.

These are involved in more complex conversions utilising the enzymatic power of these organisms (e.g. amylases for starch conversion, proteases for protein breakdown (structural changes), and lipases for degradation of lipids (e.g. in blue mould cheese)).

However, these organisms (or closely related strains) are often producing mycotoxins, which are identified as potent carcinogens. The production strains for producing e.g. koji are not producing mycotoxins on the typical substrate, (rice, soy beans etc), but if you suddenly decide to produce koji on e.g. barley, it is important to investigate if this change in substrate affects the mycotoxin production !!!!

As a side note: The fungus behind Tempeh, *Rhizopus oligosporus* do not produce mycotoxins ☺



Yeast: Cytology, taxonomy and physiology

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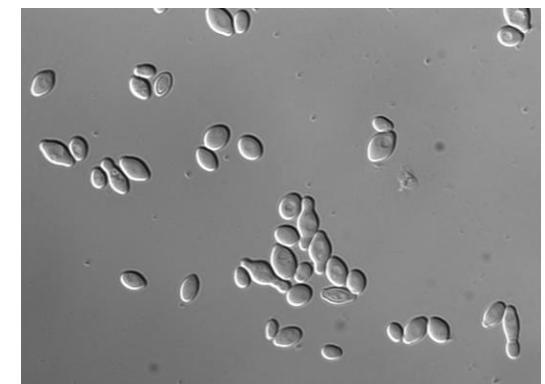
UNIVERSITY OF COPENHAGEN



Intended leaning outcome:

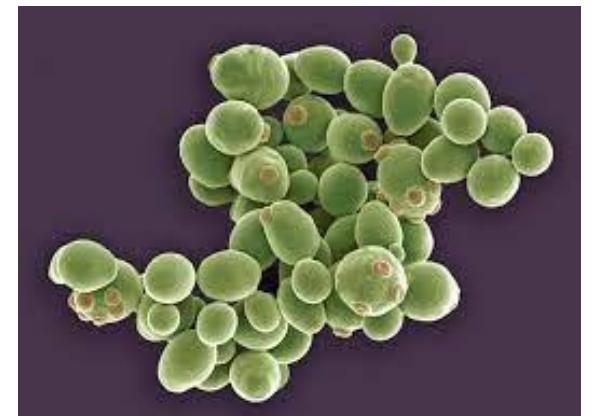
- ✓ You should be able to understand in detail the differences between bacteria (prokaryotes) and yeasts (eukaryotes)
- ✓ You should be able to understand the basic principles behind the taxonomy of yeasts
- ✓ You should have the skills to plan how to identify a given yeast isolates by use of the current taxonomy and relevant conventional and non-conventional identification techniques

Tell me your name, please....!



Background:

- Yeasts differ in several aspects from bacteria which will influence their identification, their growth characteristics as well as the interactions with the food matrix
- Proper identification of yeasts is important for the correct use of starter cultures as well as for spoilage yeasts

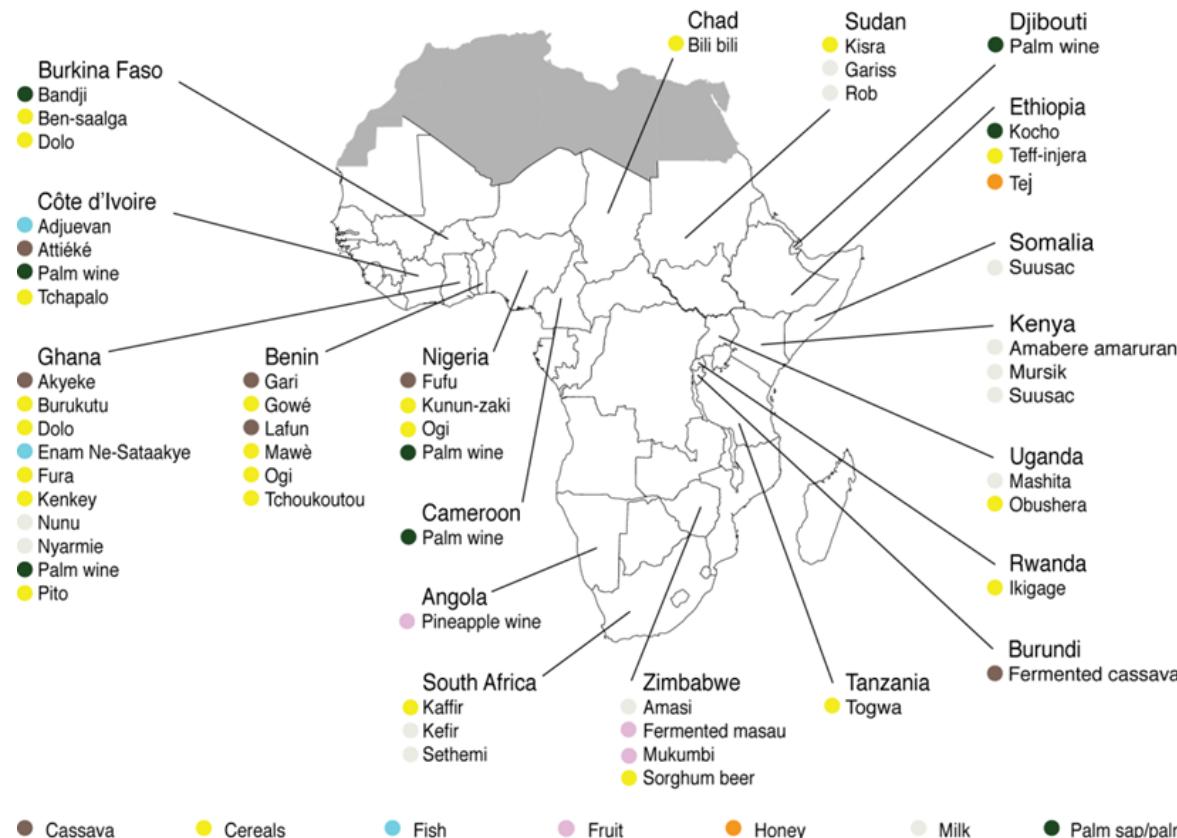


Yeasts are something we share...!



Establishment of a global awareness on species identification and strain typing

Yeasts in spontaneously fermented food and beverages produced in sub-Saharan Africa



Even spontaneously yeasts add globally to the processing of raw materials into sustainable foods

In total:

98 different yeast species identified in sub-Saharan fermented food and beverages

Most predominant yeast species:

- *Saccharomyces cerevisiae*
- *Pichia kudriavzevii (C. krusei)*
- *Candida tropicalis*
- *Kluyveromyces marxianus (C. kefyr)*

Strain variations exist



REVIEW
published: 06 August 2019
doi: 10.3389/fmicb.2019.01789



Occurrence and Importance of Yeasts in Indigenous Fermented Food and Beverages Produced in Sub-Saharan Africa

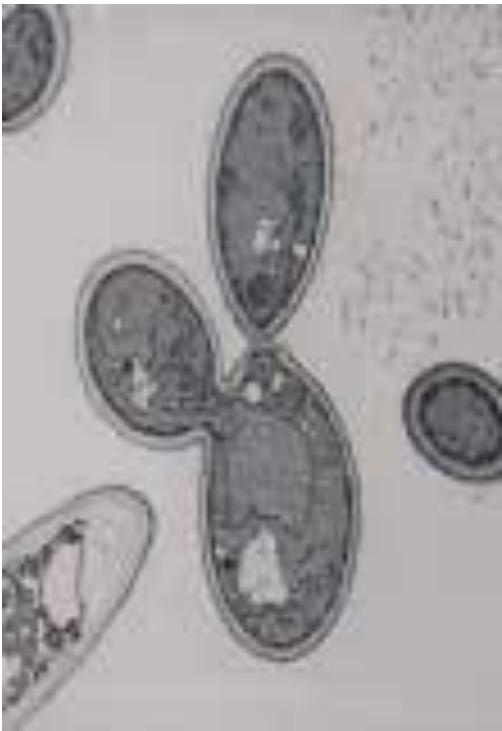
Perille Greve Johansen¹, James Owusu-Kwarteng², Charles Parkouda², S. Wilfrid Padonou⁴ and Lene Jespersen^{1*}

OPEN ACCESS

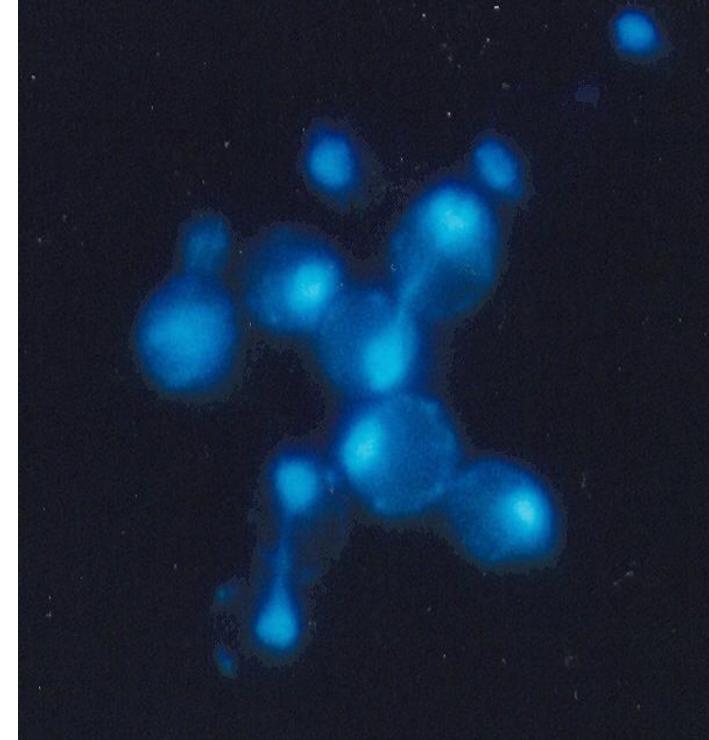
Eukaryotes

- Eukaryotes contain far more DNA than prokaryotes (x 100-1000). Yeast genome sizes range from 10-15 Mb and encode 5000-10000 genes
- The DNA is organised in a number of chromosomes
- The chromosomes are organised in a nucleus
- The presence of mitochondria
- The presence of different organelles such as: the Golgi apparatus and the endoplasmic reticulum
- Besides partly being involved in killer toxin production, the function of plasmids in yeasts is not really known
- a.o.

Yeasts are eukaryotes



Scanning electron microscopy
Saccharomyces cerevisiae



DNA staining
Saccharomyces pastorianus

Organelles

CW: cell wall (physical protection, adhesion etc.)

P: periplasm

CM: plasma membrane (transport in/out of the cytoplasma)

CMI: invagination

BS: bud scar

C: cytoplasm

N: nucleus (DNA in chromosomes)

M: mitochondrion (ATP synthesis, enzymes)

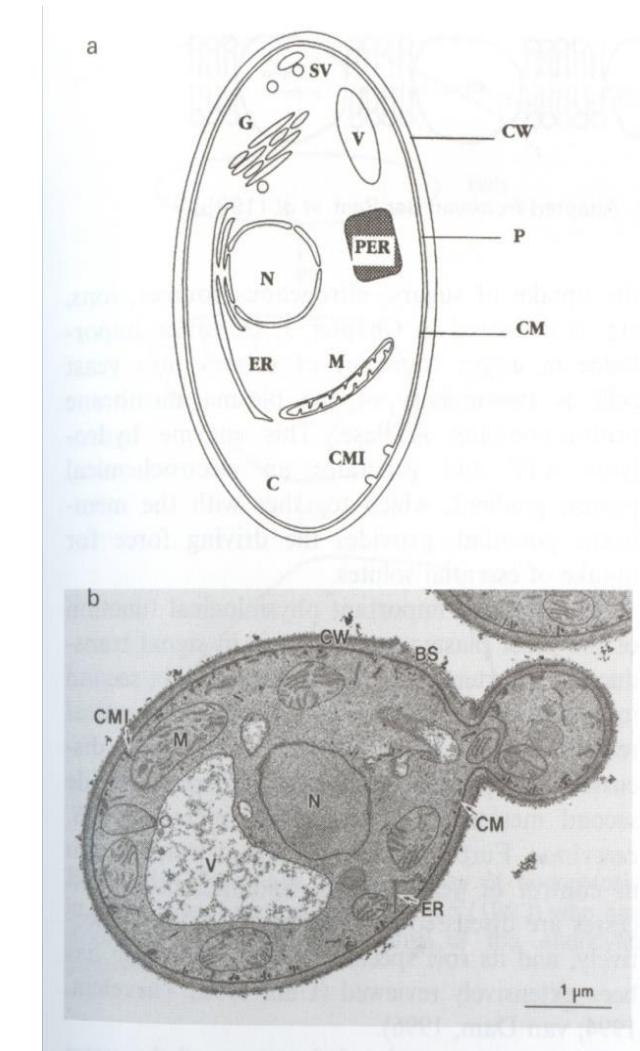
S: secretory vesicles

V: vacuole (protein trafficking, storage)

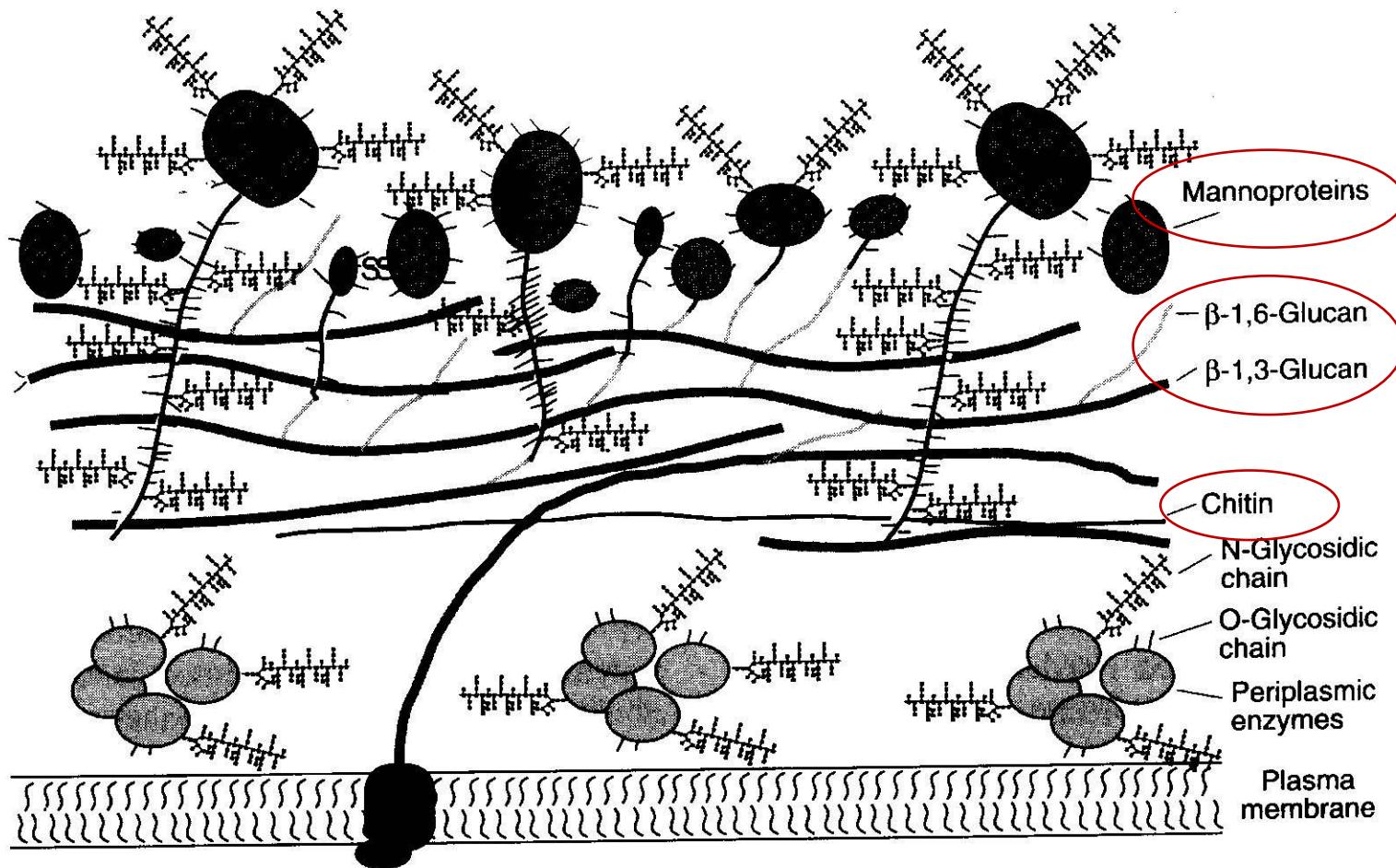
PER: peroxisome

ER: endoplasmic reticulum

G: golgi apparatus



The yeast cell wall



Physiological function of the cell wall

- Physical protection
- Maintains the shape of the yeast cell
- Osmotic stability
- Permeability barriers (solutes larger than 620-760 Da can not pass the cell wall)
- Enzyme support e.g. glucanases and hydrolases (as e.g. invertase)
- Cation binding (and binding of other toxic compounds)
- Cell-cell recognition (recognition sites for mating pheromones and killer toxins)
- Cell-cell adhesion (sexual agglutination, flocculation) and adhesion to other surfaces
including yeast-human interactions

Function – yeast plasma membrane

The primary functions of the yeast plasma membrane are to dictate what enters and what leaves the cytoplasma i.e. to be a primary barrier for passage of hydrophilic molecules and prevent cytoplasmic contents to mix with the environment

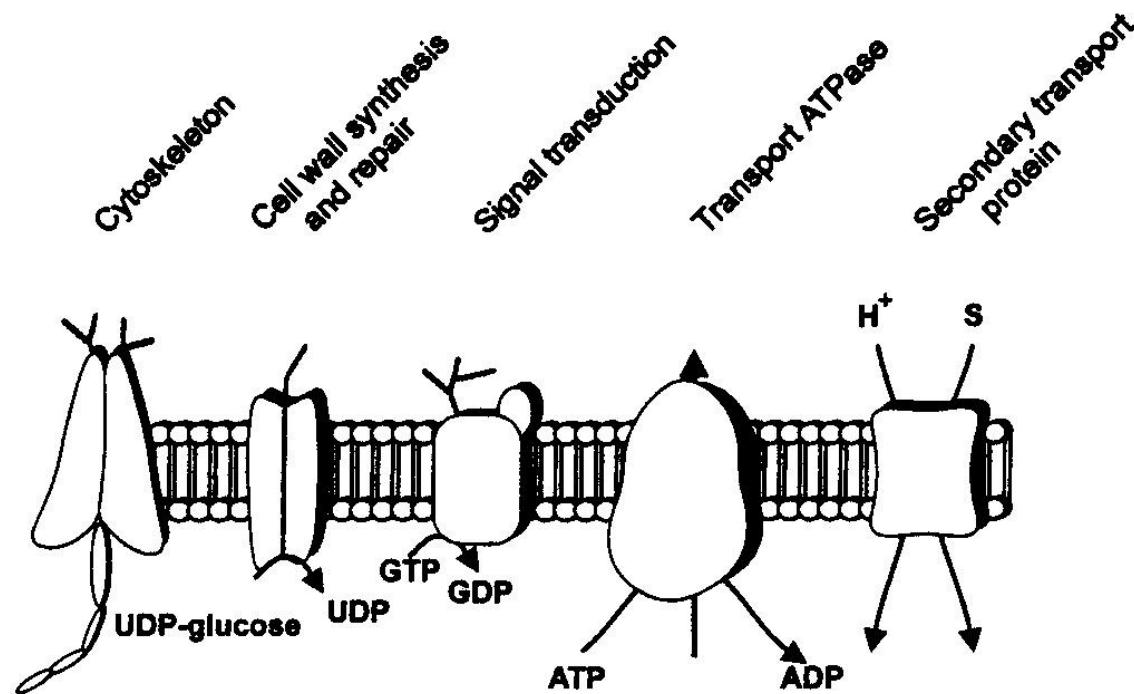
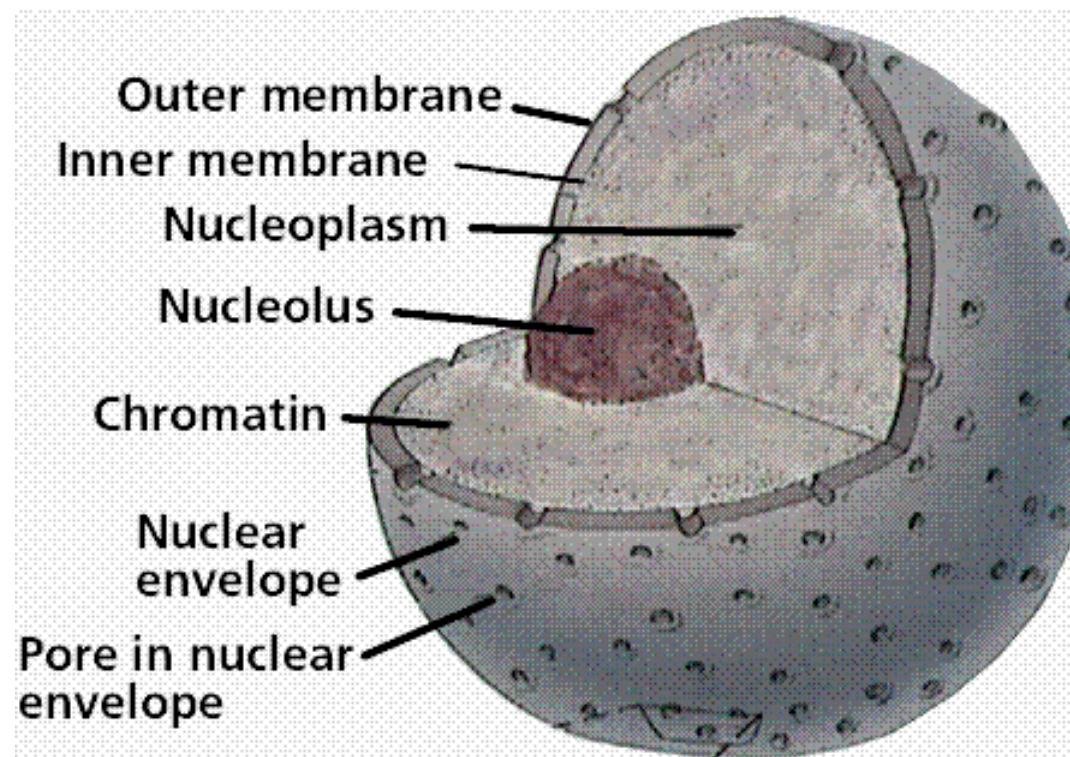


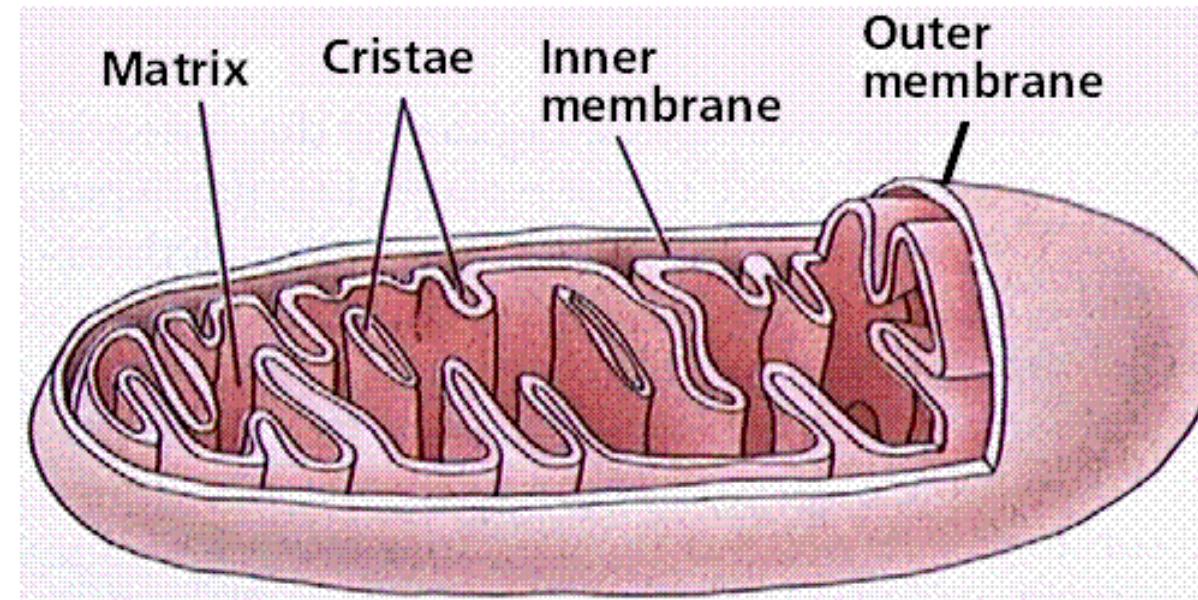
FIG. 2. Classes of membrane proteins found in the *S. cerevisiae* plasma membrane.

Yeast - nucleus



Yeast - mitochondria

Under aerobic conditions yeast mitochondria are primarily involved in ATP synthesis during respiration – under anaerobic conditions mitochondria are redundant in the respiratory sense... but have other functions

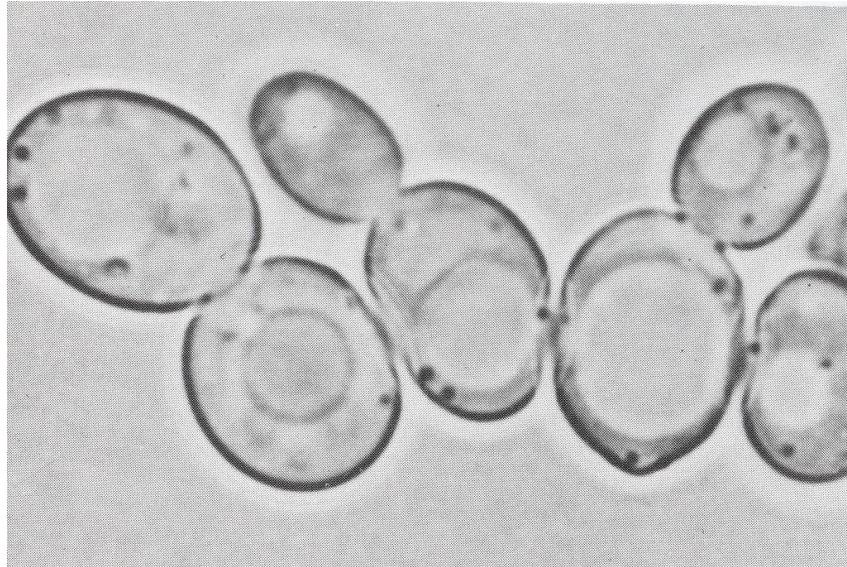


Non-respiratory functions of brewing yeast mitochondria

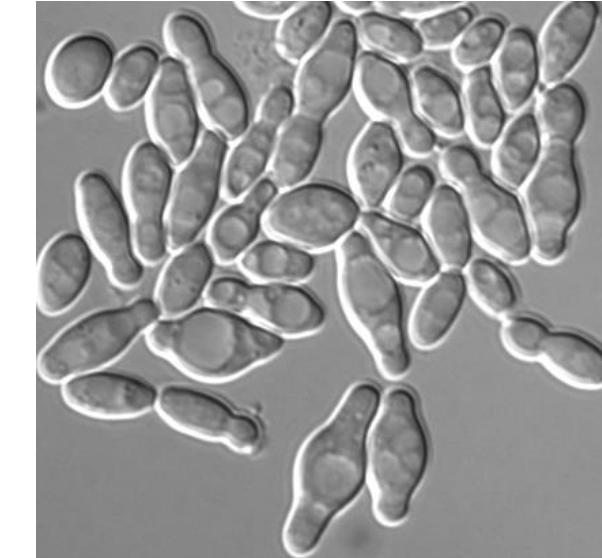
- Synthesis and desaturation of fatty acids and membrane lipids
- Mitochondrial cytochromes involved in ergosterol biosynthesis
- General physiological adaptation to stresses caused by e.g. ethanol, toxic oxygen radicals etc.
- Modification of cell surface characteristics involved in e.g. flocculation
- Production of enzymes for synthesis of amino acids, some dicarboxylic acids, pyrimidine/purine bases etc.
- Mobilisation of glycogen
- Production of flavour and aroma compounds as a detoxification process (relevant especially in beer production)

Yeast - vacuoles

The vacuole is a key organelle involved in intracellular protein trafficking in yeasts – degradation of proteins and secretion of proteases. Besides the vacuoles function as storage compartments for AS, polyphosphate and metal ions and are involved in osmoregulation



Vacuoles in *Saccharomyces cerevisiae*



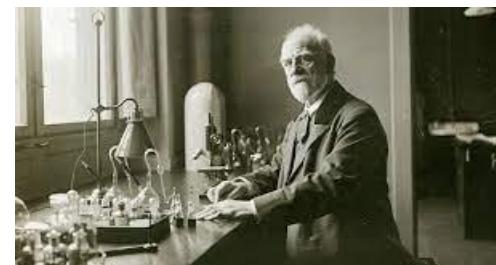
Vacuoles in *Hanseniaspora jakobsenii* sp. nov.

Definition of yeasts

Unicellular fungi reproducing by
budding or fission

I: Perfect yeast (ascomycetes and basidiomycetes)

II: Imperfect yeast (ascomycetous and basidiomycetous affinities)



Current classification

The Yeasts: a Taxonomic Study

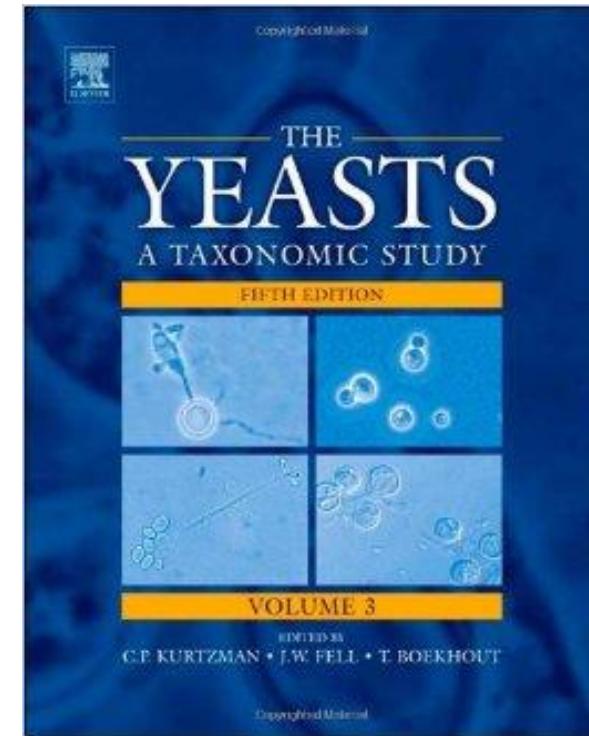
Kurtzman, C.P., Fell, J.W., and Boekhout, T. (2011)
5 ed. Vol 1-3, Elsevier, Amsterdam, NL

Yeasts: Characteristics and Identification

J.A. Barnett, R.W. Payne and Y. Yarrow (2000)
Cambridge University Press, 3rd ed. pp 1139

Today mostly databases are used e.g.:

- <https://theyeasts.org/>
- <https://www.mycobank.org/>



Taxonomy – is changeable…!

Saccharomyces

Lodder (1970):

Saccharomyces spp. > 40 species

Kreger-van Rij (1984):

Saccharomyces spp. = 7 species

Vaughan-Martini and Kurtzman (1985):

Saccharomyces cerevisiae → *Sacch. cerevisiae*

Sacch. bayanus

Sacch. pastorianus

Kurtzman and Fell (1998):

Saccharomyces spp. = 14 species

Naumov *et al.* (2000):

suggest three new species within *Saccharomyces*

Kurtzman, Fell and Boekhout (2011):

Saccharomyces spp. = 8 species

New yeast species are reported in scientific literature

International Journal of Systematic and Evolutionary Microbiology (2010), 60, 1460–1465

DOI 10.1099/ijs.0.016006-0

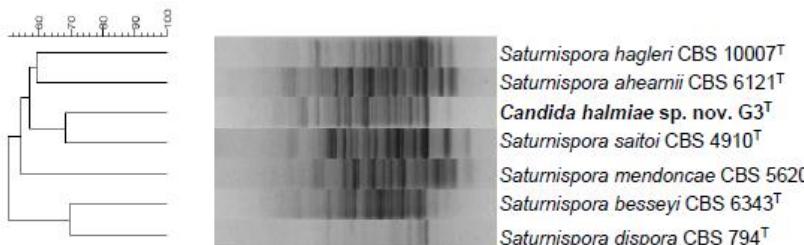
Exploring new yeast species in fermented cocoa beans from Ghana

Candida halmiae sp. nov., *Geotrichum ghanense* sp. nov. and *Candida awuaïi* sp. nov., isolated from Ghanaian cocoa fermentations

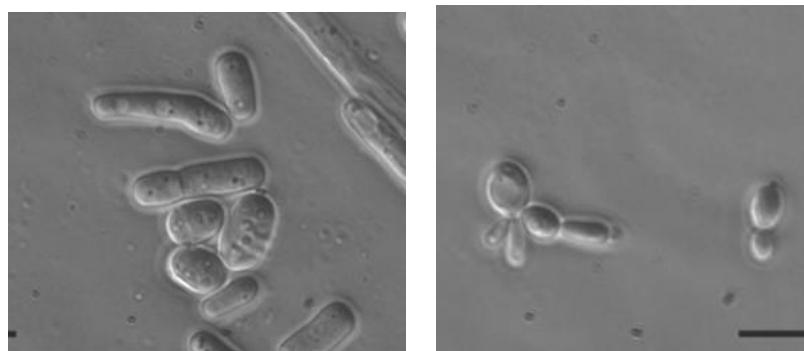
Dennis S. Nielsen, Mogens Jakobsen and Lene Jespersen

Department of Food Science, Food Microbiology, Center for Advanced Food Studies (LMC), Faculty of Life Sciences, University of Copenhagen, Denmark

During an investigation of the microbiology of Ghanaian cocoa fermentations, a number of yeast



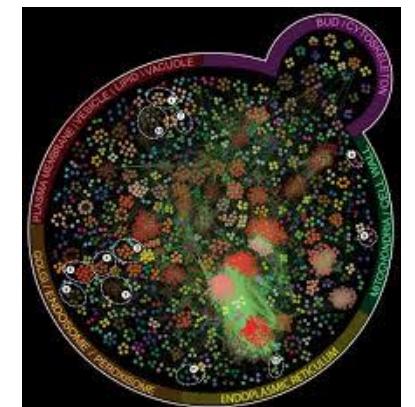
Supplementary Fig. S1. (GTG)₅-PCR fingerprints and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (*r*, expressed as a percentage value for convenience) of isolate G3^T and phylogenetic closest relatives.



Methodologies for **species identification** of yeasts

- Micro- and macromorphological identification added phenotypic characterisation
- Sequencing of the D1/D2 domain of the 26S rRNA gene (> 99% homology = belong to the same species)
- Sequencing of multiple genes
- Sequencing of the ITS region (mostly moulds and yeasts identified by metagenomics)
- Identification of protein patterns by MALDI-TOF MS
- Analysis of biomass by Fourier Transform Infrared Spectroscopy (FTIR)
- Others

<https://theyeast.org/>



From species to strain level: strain diversity within *P. kudriavzevii* (*C. krusei*)

M. Houngbédji et al.

Food Microbiology 76 (2018) 267–278

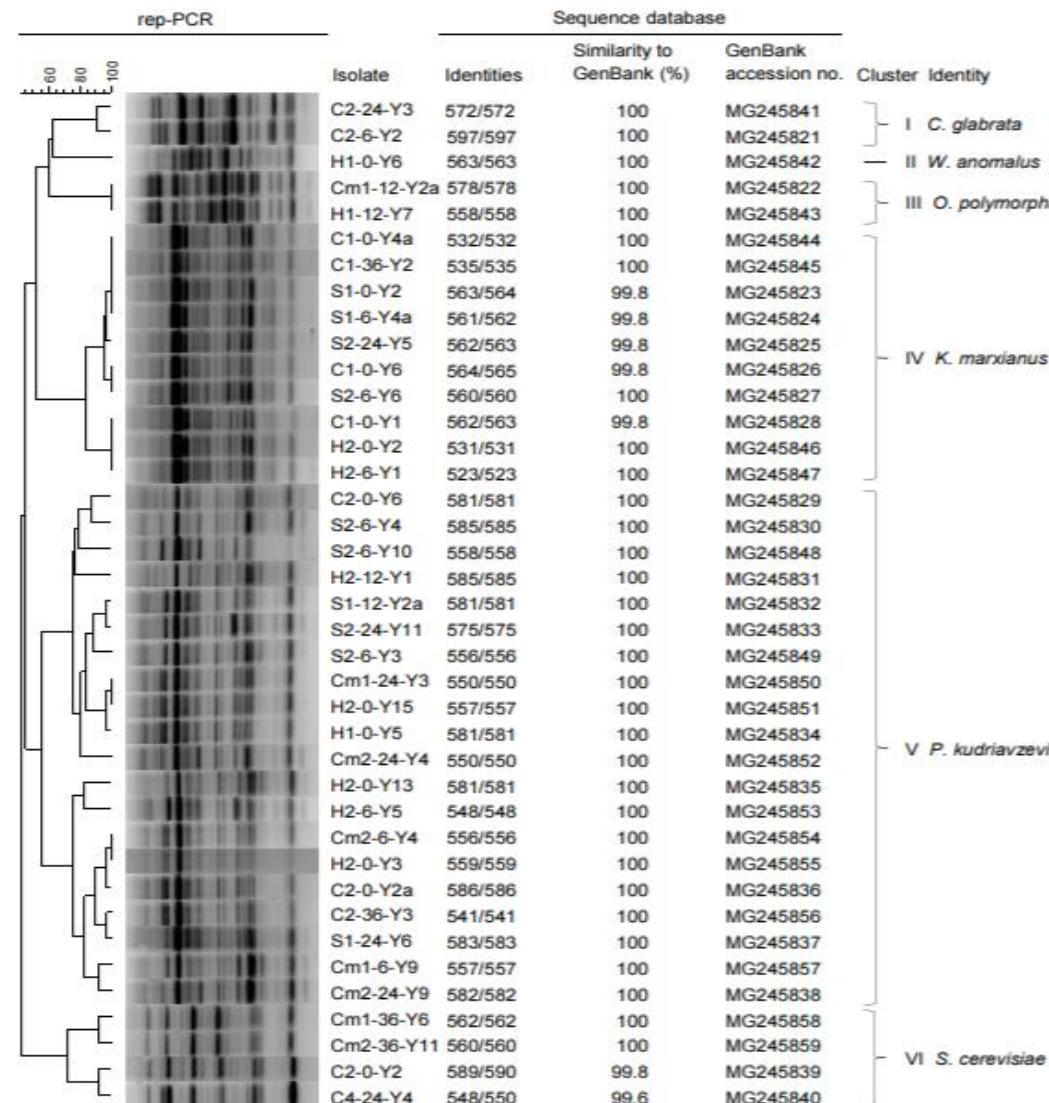


Fig. 3. Species identification of yeasts isolated during spontaneous fermentations of the four different types of mawé at two separate production sites. Dendrogram obtained by cluster analysis of (GTG)₅-based rep-PCR fingerprints of representative yeasts, based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA). Yeast species were identified by sequencing of 26S rRNA gene and GenBank searches.

Food Microbiology 76 (2018) 267–278

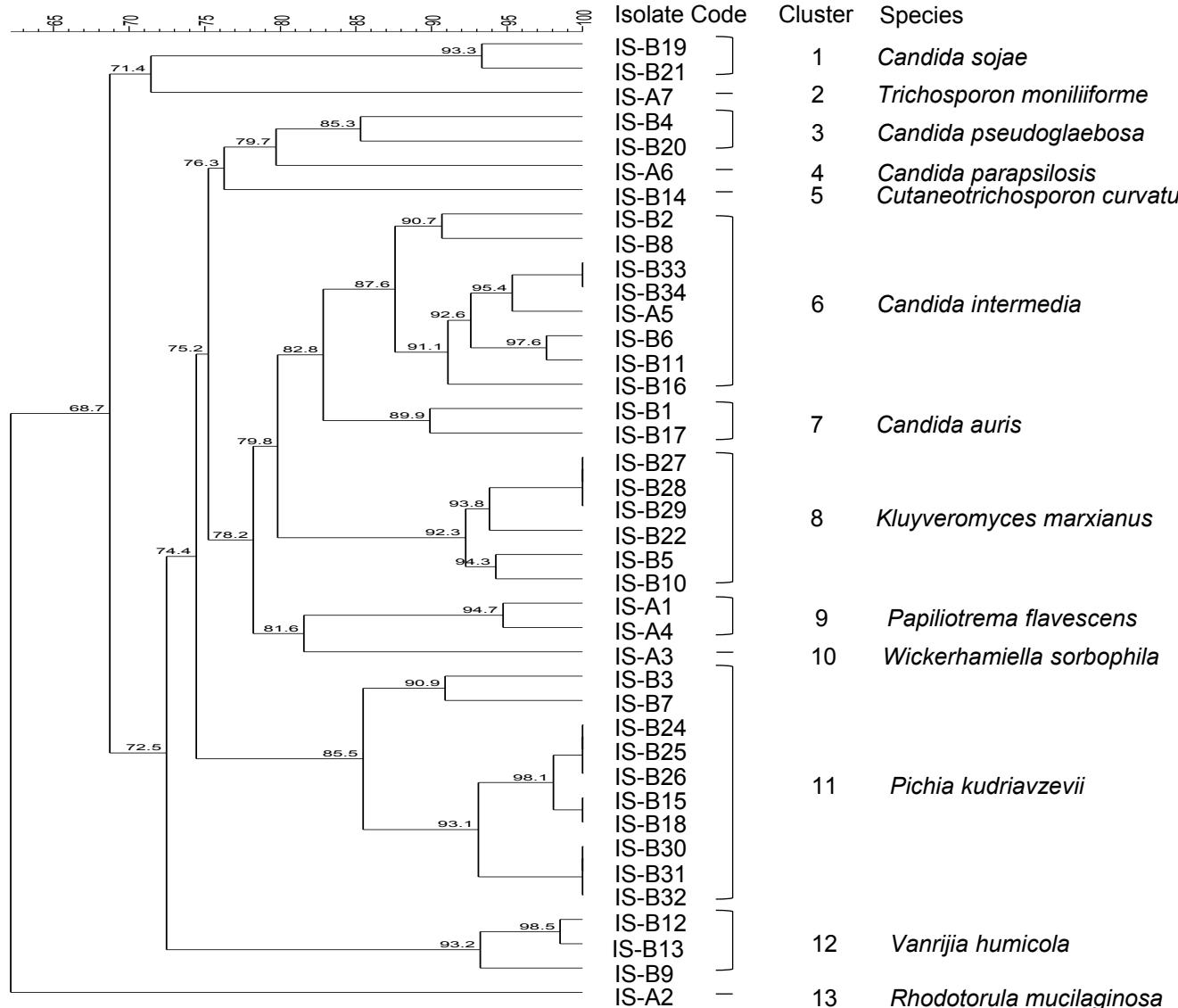
Contents lists available at ScienceDirect



Food Microbiology

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

Spoilage yeasts found in Danish white brine cheeses during production



Occurrence of Yeasts in White-Brined Cheeses: Methodologies for Identification, Spoilage Potential and Good Manufacturing Practices

Athina Geronikou¹, Thanayarn Srimahaek¹, Kalliope Rantsiou¹, Georgios Triantafyllidis³, Nadja Larsen^{1*} and Lene Jespersen¹

¹ Department of Food Science, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark, ² Department of Agricultural, Forestry and Food Sciences, University of Turin, Turin, Italy, ³ Jots S.A., Food Industry, Athens, Greece



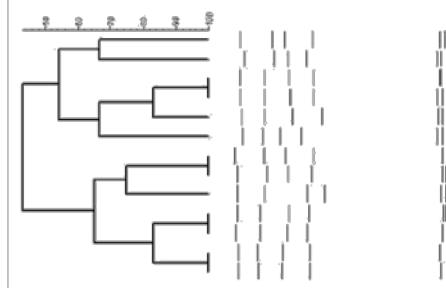
Yeasts isolated from old curd

Dendrogram of (GTG)₅-PCR fingerprint of yeast isolates from the production line of white-brined cheese, based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA).

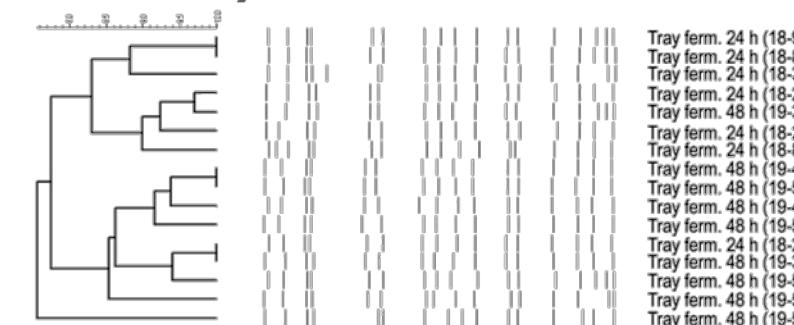
Yeasts in cocoa fermentation – biodiversity at the strain level

Subspecies typing (PFGE) of yeast species predominant during fermentation of cocoa beans

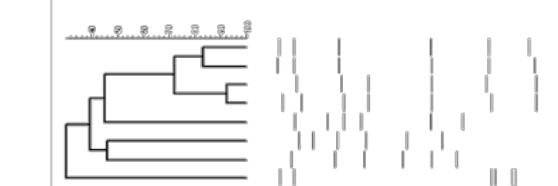
Candida krusei



Saccharomyces cerevisiae



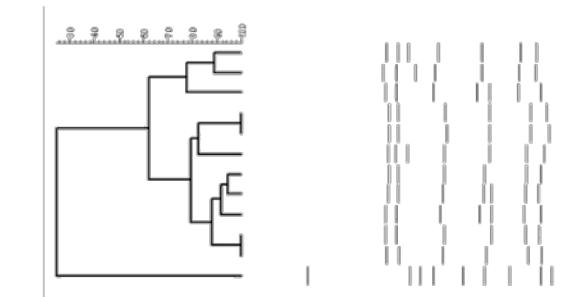
Pichia kluyveri



Pichia membranifaciens



Hanseniaspora guilliermondii



Cocoa beans – the playground for microbiologists



Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans

Lene Jespersen *, Dennis S. Nielsen, Susanne Hennholt, Mogens Jakobsen

Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University,
Bogstadvej 30, 1875 Frederiksberg C, Copenhagen, Denmark

FEMS
Yeast Research

www.fems-microbiology.org

Let's take a break!

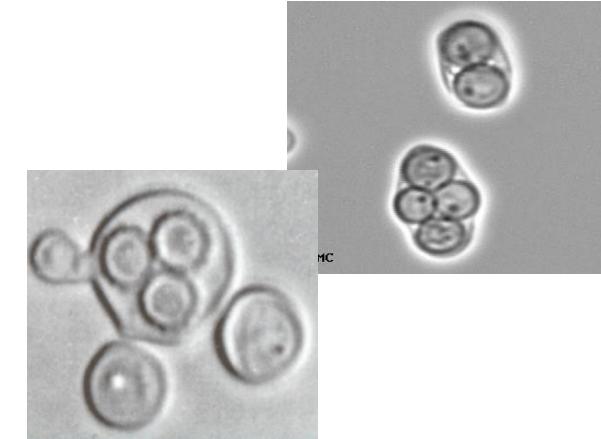


Classification of yeasts in the Eumycota

PHYLUM

Ascomycota

- teleomorphic (perfect) ascomycetous yeasts
- anamorphic (imperfect) ascomycetous yeasts



Basidiomycota

- teleomorphic basidiomycetous yeasts
- anamorphic basidiomycetous yeasts



Beloved yeasts have many names...

32.10. *Hanseniaspora valbyensis* Klöcker (1912b)

Anamorph: *Kloeckera japonica* Saito & Ohtani

Synonyms:

Endomyces valbyensis (Klöcker) Zender (1925a)

Kloeckera japonica Saito & Ohtani (1931)¹

Kloeckera corticis (Klöcker) Janke var. *pulquensis* Ulloa & Herrera (1973)¹

¹ Synonymy determined by DNA reassessments (Meyer et al. 1978).

Growth on glucose-peptone-yeast extract agar: After 1 month at 25°C, the streak culture is white to cream colored, smooth, glossy, and slightly raised at the center.

Growth in glucose-peptone-yeast extract broth: After 2 days at 25°C, the cells are apiculate and spherical, ovoid or elongate, 2–5.5 × 3–10.2 µm, and occur singly or in pairs (Fig. 32.6). Sediment is present. After 1 month a thin ring may be formed.

Dalmau plate culture on potato agar: Poorly developed branched pseudohyphae may be present or absent.

Formation of ascospores: Usually two, but occasionally four hat- to helmet-shaped ascospores are formed per ascus and they are usually released at maturity. Liberated ascospores often aggregate. Ascospores were observed on 5% Difco malt extract agar and potato dextrose agar after 7 or more days at 25°C (Fig. 32.7).

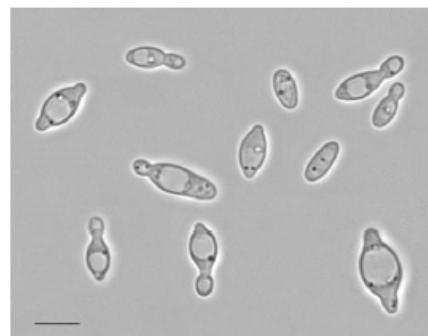


FIGURE 32.6 *Hanseniaspora valbyensis* CBS 479. Budding cells in Yeast Nitrogen Base with glucose. Bar = 5 µm (CBS website, T. van Beers and T. Boekhout).

34.29. *Kazachstania unispora* (Jörgensen) Kurtzman (2003)

Synonyms:

Saccharomyces unisporus Jörgensen (1909)

Saccharomyces mongolicus Naganishi (1928)

Growth on 5% malt agar: After 1 month at 20°C, the streak culture is cream, flat, usually glossy, smooth, sometimes with light striations, and with an entire or undulating margin.

Growth in 5% malt extract: After 3 days at 25°C, the cells are globose to short ellipsoidal, 2.5–4.5 × 3–6 µm, and occur singly or in pairs. Sediment is present. After 1 month at 20°C, sediment and a ring are present.

Growth on the surface of assimilation media: Pellicles were not formed after 21 days at 25°C.

Dalmau plate culture on morphology agar: Pseudohyphae were not formed after 5 days at 25°C.

Formation of ascospores: Budding cells are transformed directly into asci containing one, or occasionally two, globose to subglobose ascospores (Fig. 34.25). Sporulation was observed on McClary's acetate and YM agars after 1–10 days at 25°C.

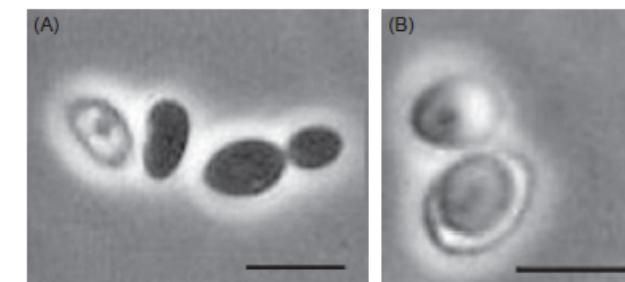


FIGURE 34.25 *Kazachstania unispora* DBVPG 6482. (A) Budding cells after 24 hours on YEPG agar at 25°C. (B) An unconjugated ascus with an ascospore after 24 hours on McClary's acetate agar at 25°C. Bars = 5 µm.

Substrates for yeasts

- Malt Yeast extract Glucose Peptone agar (MYGP)
- Yeast extract Peptone Glucose (YPG) agar
- Malt Agar (MA)
- Lysine agar a.o. (for non-Saccharomyces yeasts)
- WLN medium (allows separation by coloration)
- Etc.

Media for stress tolerant yeasts

Purpose	Media
Moderately xerotolerant yeasts	Yeast nitrogen base + sodium chloride (10% (w/v)) + glucose (5 (w/v))
Xerotolerant yeast	50% (w/v) glucose-yeast extract agar
Xerotolerant yeast from foods of high salt and sugar	Wort agar + sucrose (3.5% w/v) + glucose (1.0% w/v)
<i>Zygosaccharomyces rouxii</i>	Potato-dextrose agar + sucrose (60% w/v)
Sugar-tolerant yeast from concentrated orange juice	Glucose-citric acid-tryptone agar
Isolation and cultivation of xerotolerant yeast	Malt-extract agar + glucose (2, 20, 40, 50% w/v)

Conventional methods for identification of yeasts (1/2)

- CULTURAL CHARACTERISTICS
 - Solid media
 - Liquid media
- CELL MORPHOLOGY AND ARRANGEMENT
 - Cell form
 - Vegetative reproduction (budding/fission)
 - Pseudomycelium / true mycelium
- SEXUAL CHARACTERISTICS
 - Perfect/imperfect
 - Formation and arrangement of spores

Conventional methods for identification of yeasts (2/2)

- BIOCHEMICAL CHARACTERISTICS

- Assimilation of carbohydrates (e.g. API20c, ID32c)

- Fermentation of carbohydrates

- Assimilation of nitrate

- Etc.

- PHYSICAL AND CHEMICAL TOLERANCE

- Growth in the presence of 100 or 1000 ppm cycloheximide

- Growth in the presence of 1 % acetic acid

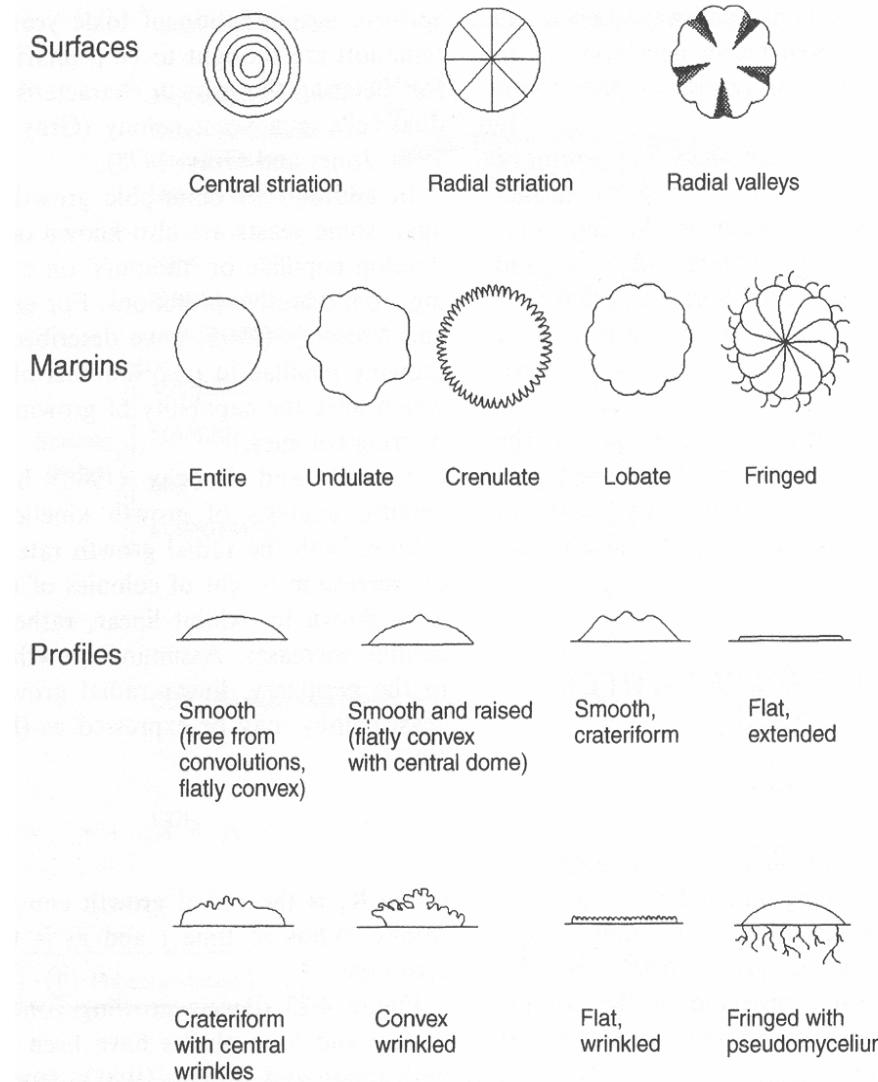
- Growth on 50 % (w/w) glucose-yeast extract agar

- Etc.

Macro-morphological characteristics (1/2)

- Texture (mucoid, viscous, matted, coherent etc.)
- Colour
- Size
- Surface (glistening, smooth, verrucose, rough etc.)
- Elevation (flat, spreading, raised etc.)
- Margin (entire, rhizoid etc.)
- Mycelium (x 100 magnification)

Macro-morphological characteristics (2/2)



Micro-morphological characteristics

- . Cell form (spheroidal, ellipsoidal, ovoid, lemon-shaped, elongated, triangular etc.)
- . Cell size (length, width)
- . Cell arrangement (pairs, aggregates)
- . Vegetative reproduction
 - Budding (monopolar, bipolar, multilateral)
 - Fission
- . Mycelium
 - Pseudomycelium (no distinct septa)
 - True mycelium (distinct septa)

Assimilation of carbon compounds (examples)

Yeast Nitrogen Base (Difco)

Hexoses

Trisaccharides

Pentoses

Organic acids

Disaccharides

Polysaccharides

Alcohols

Glycosides



Fermentation of carbon compounds (examples)

Glucose

Maltose

Trehalose

Galactose

Raffinose

Melibiose

Saccharose

Lactose

Inulin

Production of CO_2



Assimilation of nitrogen compounds (examples)

Yeast Carbon Base (Difco)

Nitrate

Ethylamine hydrochloride

L-lysine

Creatine

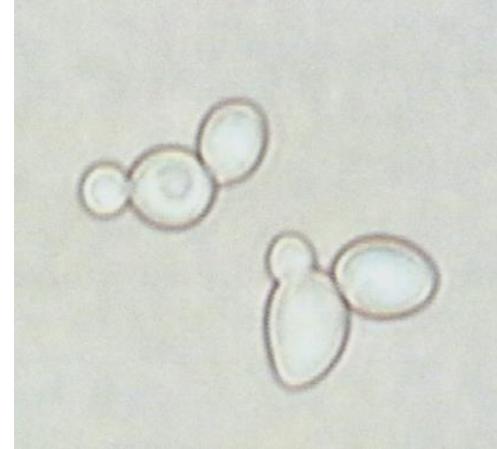
Nitrite

Cadaverine dihydrochloride

Creatinine

Vegetative reproduction

Multilateral budding



Kluyveromyces lactis (Candida spherica)

Bipolar budding

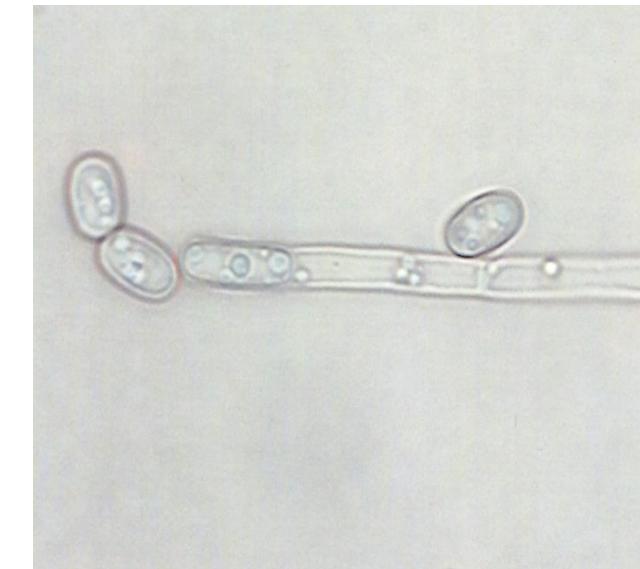


Hanseniaspora valbyensis

Fission



Schizosaccharomyces pombe



Galactomyces geotrichum

Arthroconidia

Vegetative reproduction (genera)

BIPOLAR

Hanseniaspora (*Kloeckera*)
*Rhodotorula**
Saccharomyces
Trichosporon

FISSION

Schizosaccharomyces

ARTHROCONIDIA

Galactomyces (*Geotrichum*)
*Saccharomycopsis**

ENTEROBLASTIC BUDDING

Phaffia

MULTILATERAL

Candida
Debaryomyces
Dekkera (*Brettanomyces*)
Kazachstania
Kluyveromyces
Pichia
*Rhodotorula**
Saccharomyces
*Saccharomycopsis**
Torulaspora
Yarrowia
Zygosaccharomyces

* two types of vegetative reproduction may be present

Mycelium



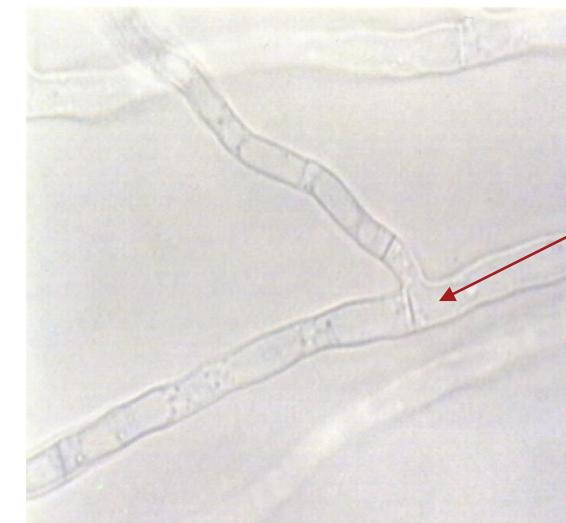
Pseudomycelium

Candida parapsilosis



Pseudomycelium

Pichia membranifaciens



True mycelium

Saccharomyces fibuligera

Formation of pseudo- and true mycelium (genera)

PSEUDOMYCELIUM

Debaryomyces
Dekkera (Brettanomyces)
Hanseniaspora (Kloeckera)
Kazachstania
Kluyveromyces
Phaffia
Saccharomyces
Saccharomycodes (poorly)
Torulaspora
Zygosaccharomyces

TRUE MYCELIUM

Galactomyces (Geotrichum)
Schizosaccharomyces
Trichosporon

PSEUDO- AND TRUE MYCELIUM

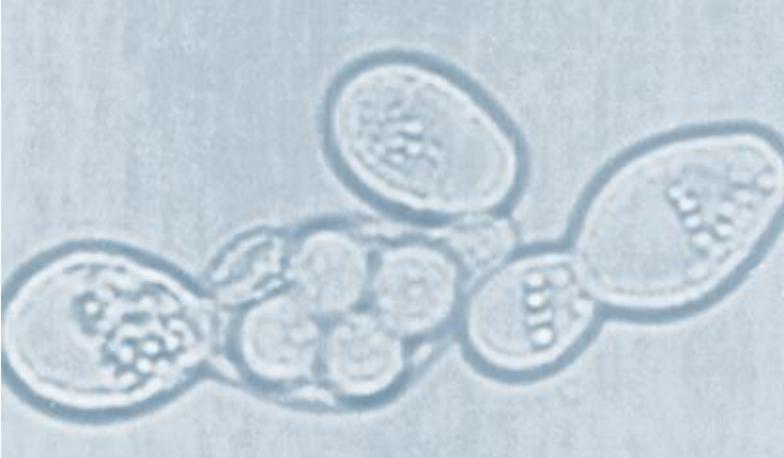
Candida
Pichia
Rhodotorula
Saccharomycopsis
Yarrowia

Sexual characteristics

- Ascosporogenous yeasts (sexual spores formed endogenously)
 - Number of spores per ascus
 - Shape of spores (hat-shaped, spherical, oval, saturn-shaped etc.)
 - Formation of asci (on hyphae, loose cells)
 - Shape of ascus
- Basidiosporogenous yeasts (sexual spores formed exogenously)
- Imperfect yeasts (anamorph)

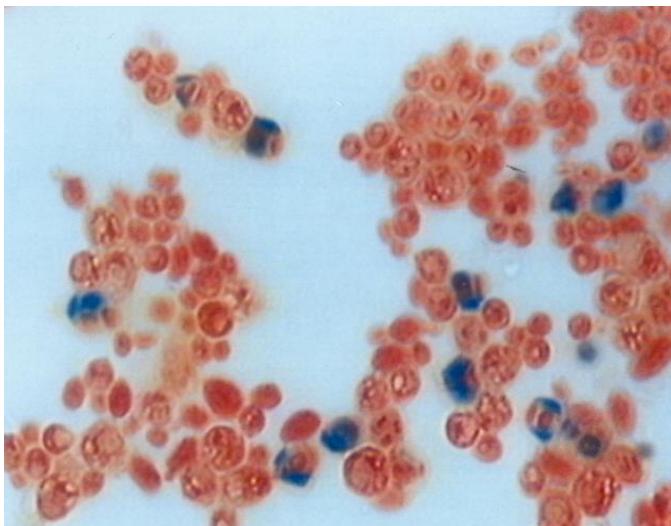
Spores

Four round spores in an ascus



Saccharomyces ludwigii

Hat-shaped spores

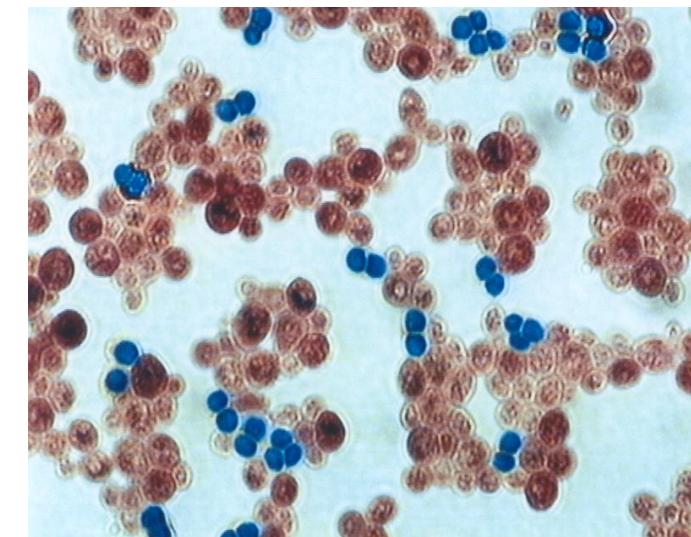


Wickerhamomyces anomalous
(*Candida pelliculosa*)



Zygosaccharomyces bailii

2 x 2 round spores and a conjugation bridge



Saccharomyces cerevisiae

Round spores

Forms of ascospores (genera)

SPHERICAL, ELLIPSOIDAL OR RENIFORM

Debaryomyces

Galactomyces (Geotrichum)

Kazachtania

Kluyveromyces

Saccharomyces

Saccharomycodes

Schizosaccharomyces

Torulaspora

Zygosaccharomyces

HAT- OR SATURNSHAPED (SPHERICAL, ELLIPSOIDAL OR RENIFORM MAY OCCUR)

Dekkera (Brettanomyces)

Hanseniaspora (Kloeckera)

Pichia

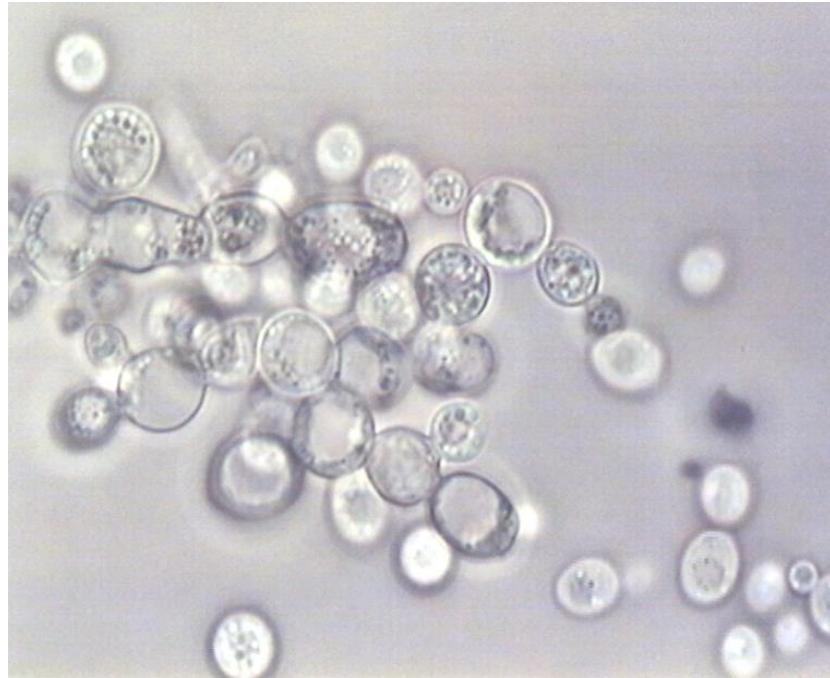
Saccharomycopsis

Yarrowia

Taxonomy of food borne yeasts

PHYLUM	FAMILY	GENUS
Ascomycota	Schizosaccharomycetaceae	<i>Schizosaccharomyces</i>
	Dipodascaceae	<i>Galactomyces</i>
	Saccharomycetaceae	<i>Yarrowia</i>
		<i>Debaryomyces</i>
		<i>Dekkera</i>
		<i>Kazachstania</i>
		<i>Kluyveromyces</i>
		<i>Pichia</i>
		<i>Saccharomyces</i>
		<i>Torulaspora</i>
		<i>Zygosaccharomyces</i>
	Saccharomycodaceae	<i>Hanseniaspora</i>
	Saccharomycopsidaceae	<i>Saccharomycodes</i>
	Candidaceae	<i>Saccharomycopsis</i>
		<i>Brettanomyces</i>
		<i>Candida</i>
		<i>Geothichum</i>
		<i>Kloeckera</i>
Basidiomycota	Sporobolomycetaceae	<i>Rhodotorula</i>
	Cryptococcaeeae	<i>Phaffia</i>
		<i>Trichosporon</i>

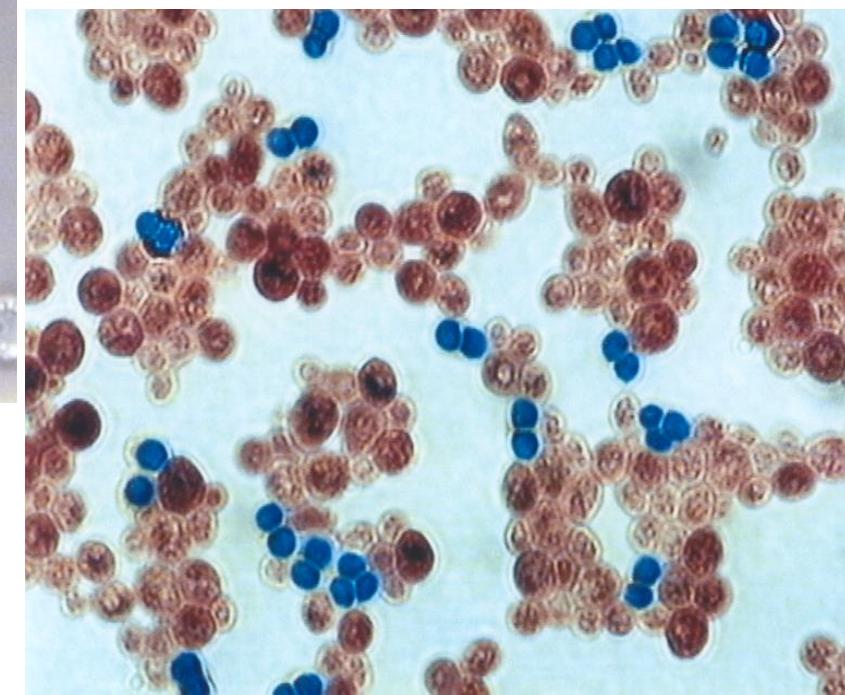
Saccharomyces cerevisiae



Multilateral budding cells



Ascus with two spores

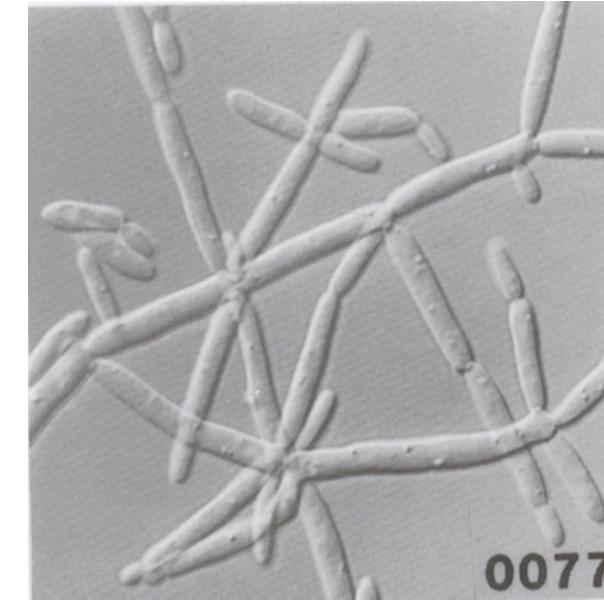


Staining of spores
S. cerevisiae var. *ellipsoideus*

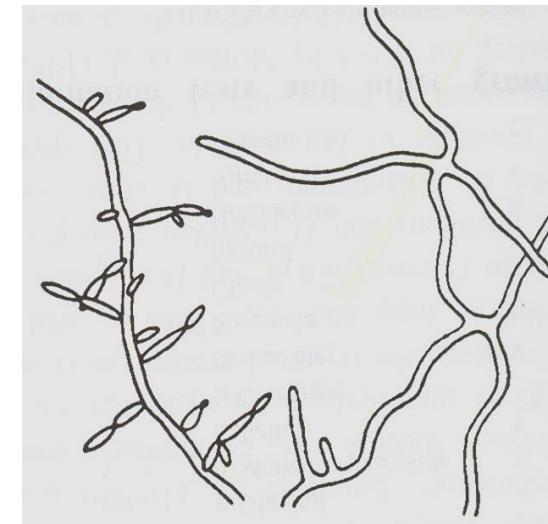
Dekkera anomala (*Brettanomyces anomalus*)



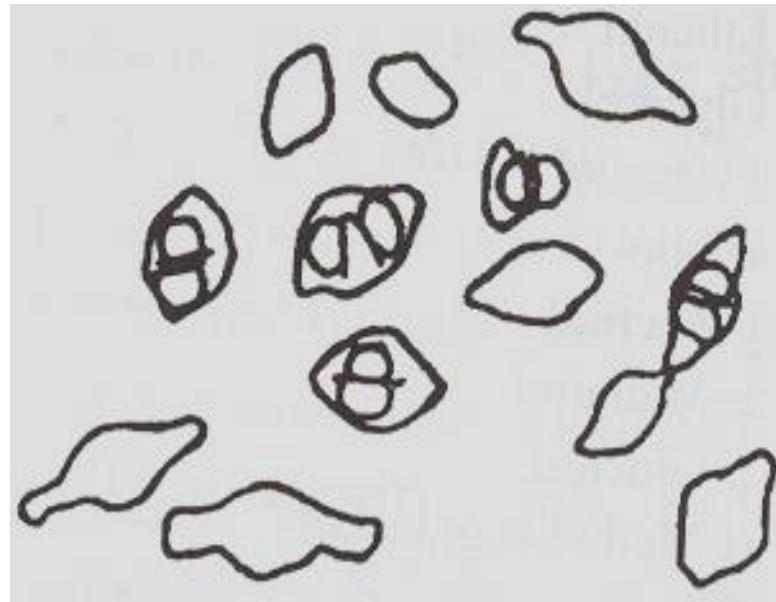
Elongated cells



Formation of pseudomycelium
(non septate filaments)



Hanseniaspora valbyensis (Kloeckera japonica)

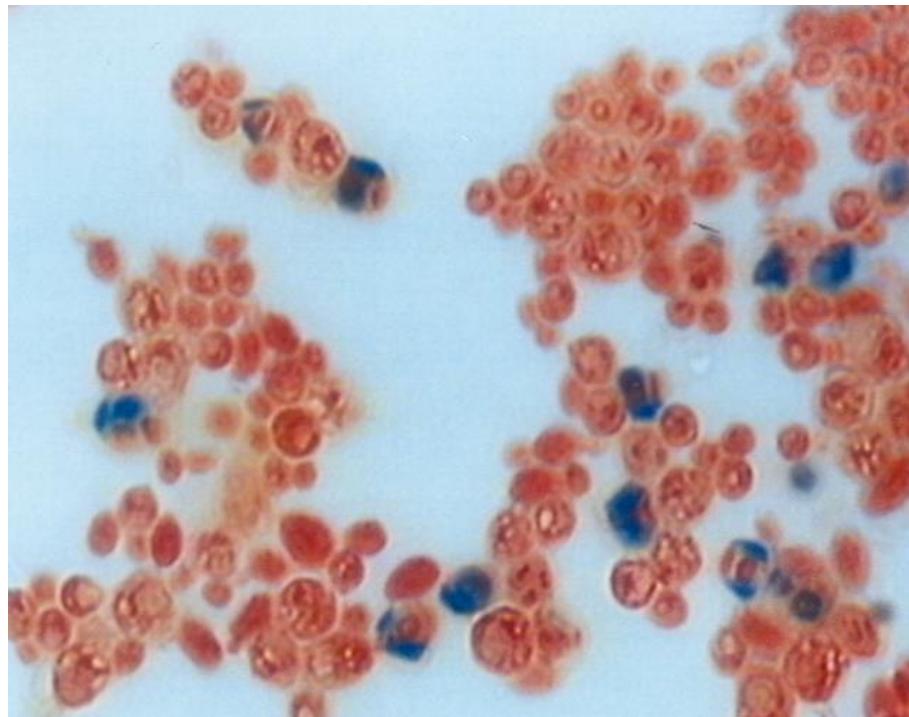


Hat-shaped spores



Bilateral budding

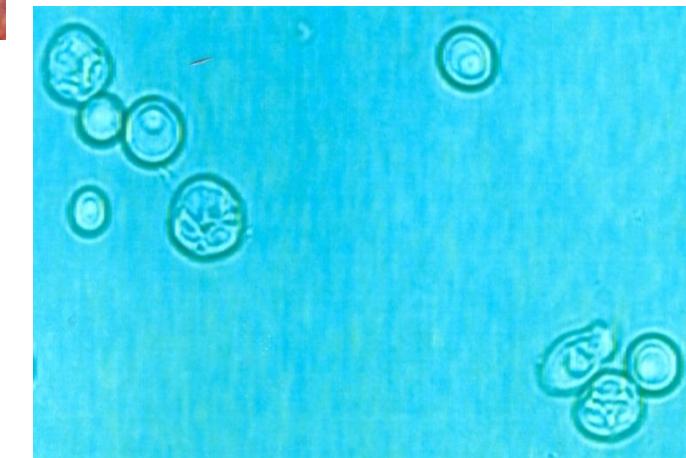
Wickerhamomyces anomalus (Candida pelliculosa)



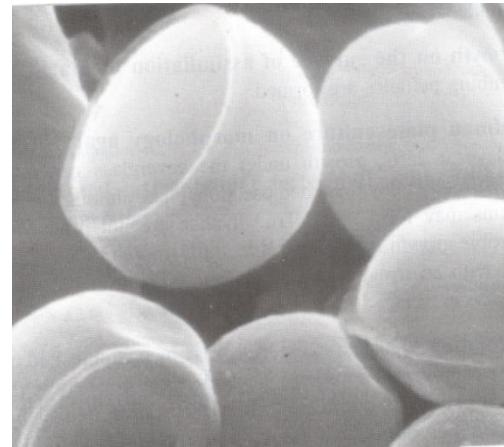
Staining of spores (hat-shaped)



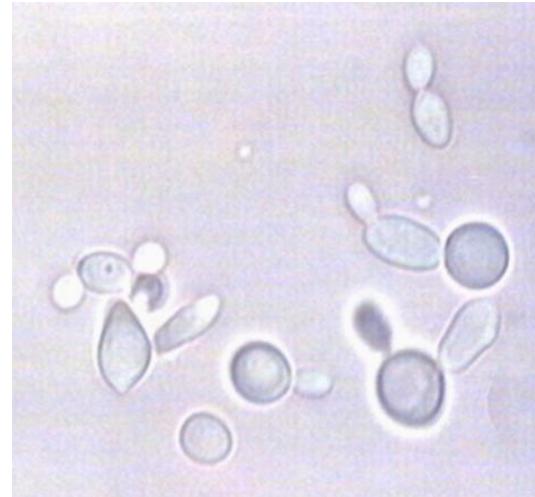
Ascus with three spores



Pichia membranifaciens (*Candida valida*)

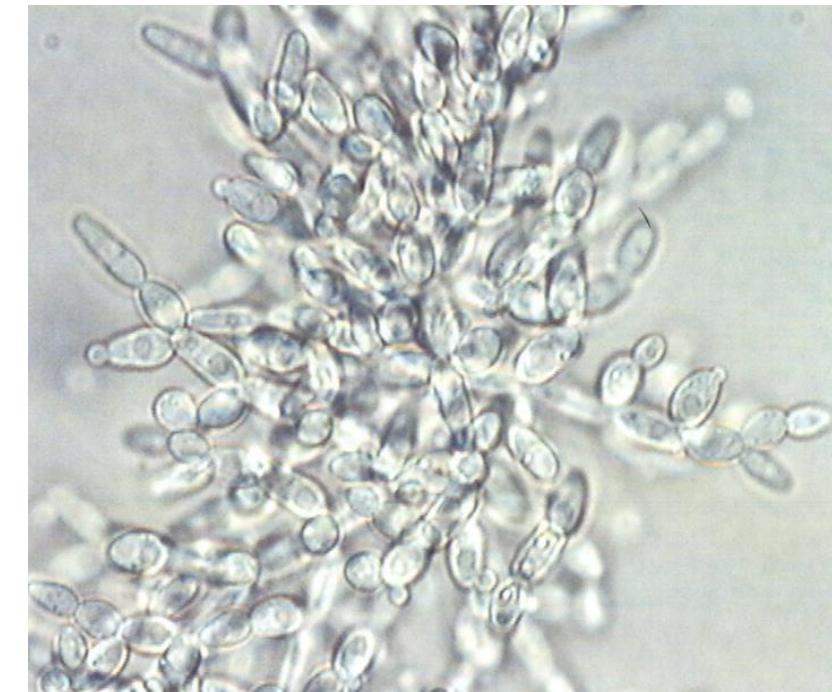


Hat- and saturn-shaped spores



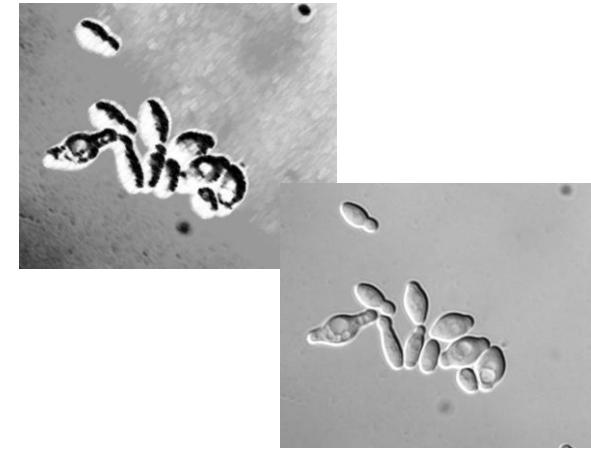
Multilateral budding

Aggregated cells /
pseudomycelium formation



In conclusion...

- ✓ Yeasts are predominantly unicellular eukaryotes
- ✓ Yeasts might have both imperfect and perfect names
- ✓ Yeast taxonomy is changing continuously especially due to new molecular techniques
- ✓ Yeasts are currently mostly identified by sequencing of the D1/D2 region of the 26S rRNA gene – however, never forget to verify by examining morphology and include phenotypic tests
- ✓ Remember to consult the newest scientific literature in order to use updated taxonomic names



LAB classification, substrates, methods, and metabolism

Paulina Deptula

Assist. Prof.

deptula@food.ku.dk



KØBENHAVNS UNIVERSITET



Intended learning outcomes

- Understanding of how we classify Lactic Acid Bacteria
- Understanding of major requirements and capabilities of LAB
- Overview of methods of detection and differentiation of LAB

Lactic Acid Bacteria

Non-taxonomic group of diverse bacteria

List of Prokaryotic names with Standing in
Nomenclature: <http://lpsn.dsmz.de>
<https://lpsn.dsmz.de/order/lactobacillales>

- Domain: *Bacteria*
 - Phylum: *Bacillota*
 - Class: *Bacilli*
 - Order: *Lactobacillales*
 - Family: *Aerococcaceae*
 - Family: *Carnobacteriaceae*
 - Family: *Enterococcaceae*
 - Family: *Lactobacillaceae*
 - Family: *Leuconostocaceae*
 - Family: *Streptococcaceae*

LPSN.dsmz.de

Subspecies *Lactobacillus delbrueckii* subsp. *bulgaricus*

Name: *Lactobacillus delbrueckii* subsp. *bulgaricus* (Orla-Jensen 1919) Weiss et al. 1984

Category: Subspecies

Proposed as: comb. nov.

Etymology: N.L. masc. adj. *bulgaricus*, Bulgarian

Gender: masculine

Type strains: ATCC 11842; CCUG 41390; CIP 101027; DSM 20081; IFO 13953; JCM 1002; LMG 13551; LMG 6901; NBRC 13953; NCTC 12712; VKM B-1923

See detailed strain information at [BacDive](#)

Conduct genome-based taxonomy at [TYGS](#)

16S rRNA gene: AJ010835 Analyse [FASTA](#) ENA NCBI

Effective publication: Weiss N, Schillinger U, Kandler O. *Lactobacillus lactis*, *Lactobacillus leichmannii* and *Lactobacillus bulgaricus*. Subjective Synonyms of *Lactobacillus delbrueckii*, and Description of *Lactobacillus delbrueckii* subsp. *lactis* comb. nov. and *Lactobacillus delbrueckii* subsp. *bulgaricus* comb. nov. *Syst Appl Microbiol* 1983; 4:552-557. [PubMed](#)

IJSEM list: Anonymous. Validation list no. 14. Validation of publication of new names and new combinations previously effectively published outside the IJSB. *Int J Syst Bacteriol* 1984; 34:270-271. [PubMed](#)

Nomenclatural status: validly published

Taxonomic status: correct name

Risk group: 1

Synonyms:

Name	Kind
<i>Lactobacillus bulgaricus</i> (Orla-Jensen 1919) Rogosa and Hansen 1971	homotypic synonym, validly published, distinct rank

Parent taxon: *Lactobacillus delbrueckii* (Leichmann 1896) Beijerinck 1901 (Approved Lists 1980)

Assigned by: Weiss N, Schillinger U, Kandler O. *Lactobacillus lactis*, *Lactobacillus leichmannii* and *Lactobacillus bulgaricus*. Subjective Synonyms of *Lactobacillus delbrueckii*, and Description of *Lactobacillus delbrueckii* subsp. *lactis* comb. nov. and *Lactobacillus delbrueckii* subsp. *bulgaricus* comb. nov. *Syst Appl Microbiol* 1983; 4:552-557. [PubMed](#)

Lactic Acid Bacteria: Definition

- Gram positive
- Non-spore formers
- Catalase negative
- Obligatory fermentative
- Lactic acid is major end product in glucose fermentation
- Usually non-motile
- Most have extensive growth requirements, but also capabilities

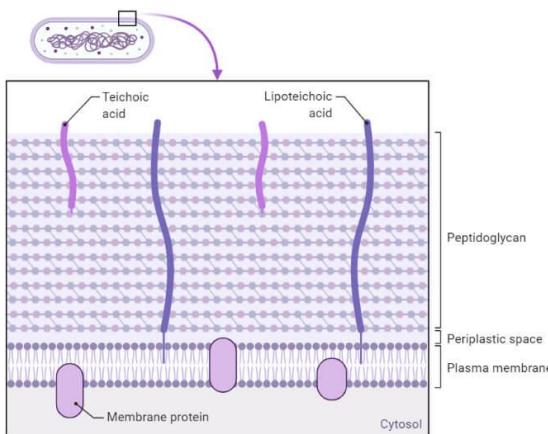
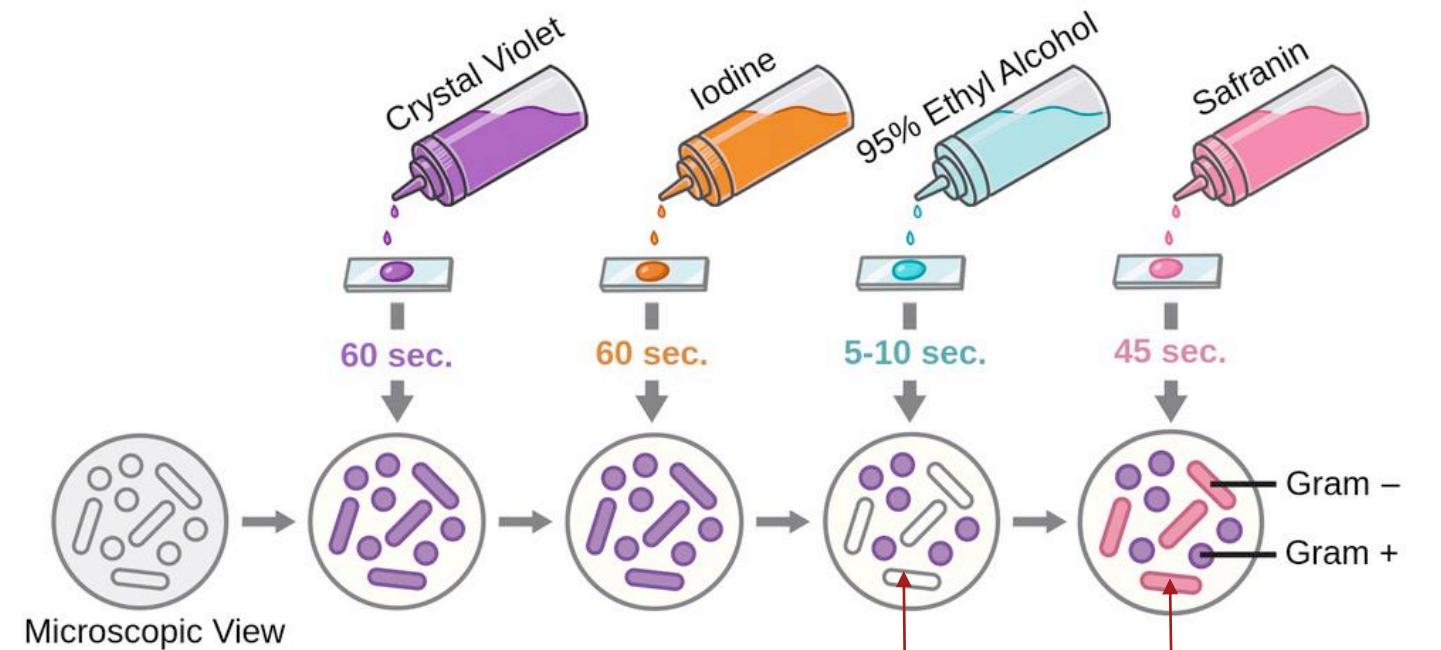
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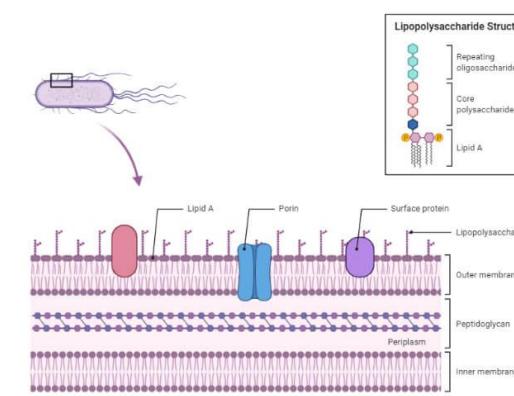
Gram staining



Hans Christian Gram
1853-1938
Danish bacteriologist



Gram-Positive Bacteria Cell Wall Structure



Gram-Negative Bacteria Cell Wall Structure

Alcohol washes out crystal violet from thin peptidoglycan layer of Gram -

Staining with safranin is visible

- **Gram positive**

- Thick layer of peptidoglycan = thicker, more rigid cell wall
- No outer membrane

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Non-spore formers

“**Bacterial spores** are a highly successful type of **dormant cell**. Sporulation provides a mechanism by which spore-formers can **withstand unfavorable conditions, at temporal and/or spatial scale**. Spores are both resistant structures that can **persist** in a dormant state for extended periods of time, and a vector for **increasing dissemination rate**, both circumventing local unfavorable conditions that prevent optimal growth.”

Paul, Christophe, et al. "Bacterial spores, from ecology to biotechnology." *Advances in applied microbiology* 106 (2019): 79-111.

- Domain: *Bacteria*
 - Phylum: *Bacillota*
 - Class: *Bacilli*
 - Order: *Lactobacillales*
 - Family: *Aerococcaceae*
 - Family: *Carnobacteriaceae*
 - Family: *Enterococcaceae*
 - Family: *Lachnospiraceae*
 - Family: *Leuconostocaceae*
 - Family: *Streptococcaceae*
 - *Bacillales*
 - *Bacillaceae*
 - *Bacillus*
 - *Bacillus subtilis, Bacillus cereus*
 - *Clostridia*
 - *Eubacteriales*
 - *Clostridiaceae*
 - *Clostridium*
 - *Clostridium tyrobutyricum, Clostridium botulinum, Clostridium estericum*

These bacteria produce endospores

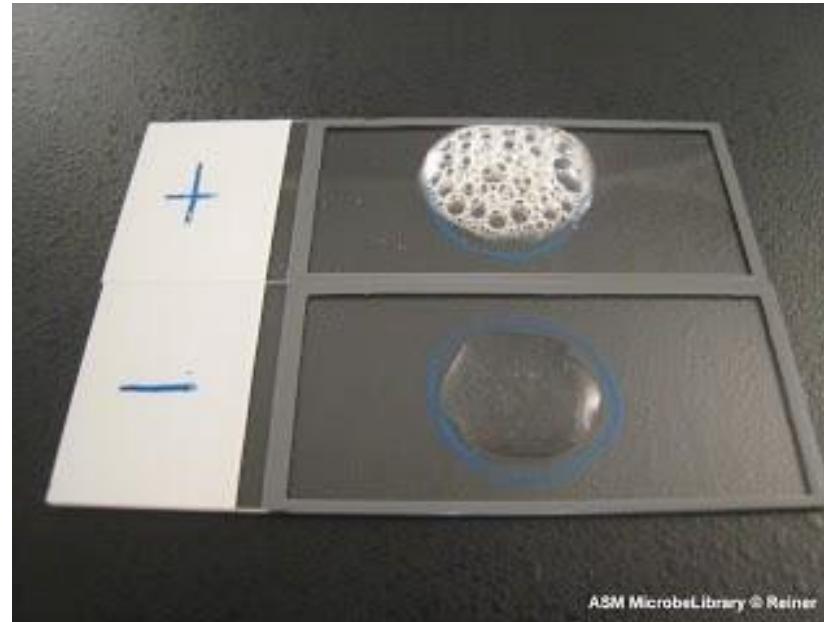
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Catalase test

Hydrogen Peroxide
Degradation
(to protect the cells from
oxidative stress)

Catalase activity
 $2 \text{ H}_2\text{O}_2 \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$



- A) LAB are catalase negative, i.e. no bubbles are formed with hydrogen peroxide exposure
- B) Some LAB e.g. *Latilactobacillus sakei* has pseudocatalase activity when grown with Heme-groups (i.e. on blood agar)
- C) Few LAB have true catalase activity and usually low e.g. few strains of *Lactiplantibacillus plantarum*

Lactic Acid Bacteria: Definition

- Gram positive
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- **Obligatory fermentative**
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Obligatory fermentative bacteria

- Can only generate energy through fermentation of carbon sources as opposed to aerobic respiration, which requires oxygen as electron acceptor
- Not to be confused with inability to grow in presence of oxygen.
→ LAB are facultative anaerobes (i.e. can grow in presence of oxygen, but do not require it)
- LAB division based on types of fermentation:
 - Homofermentative
 - Heterofermentative

2 types of LAB fermentation

- **Homofermentative**

- Ferment hexoses (e.g. glucose, galactose, fructose) to lactic acid using homofermentative **Embden-Meyerhoff-Parnass (EMP)** pathway
- Lactic acid as major end product

CO₂ production test on glucose

- **Heterofermentative**

- Ferment hexoses and pentoses (e.g. ribose, arabinose, xylose) using the heterofermentative **phosphoketolase** pathway
- in addition to lactic acid, **CO₂** and acetic acid/ethanol as major end products

- Older division included "Facultative heterofermentative"

- Ferment glucose to lactic acid using homofermentative pathway
 - Ferment pentoses (if fermented) using the heterofermentative pathway.
- Abolished, because pentose fermentation varied even within species, complicating classification

More in LAB physiology lecture



Lactic Acid Bacteria: Definition

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- Lactic acid is major end product in glucose fermentations
- Usually non-motile
- **Most have extensive growth requirements, but also capabilities**

LAB growth requirements

- **Fastidious** bacteria i.e. require multiple nutrients for growth:
 - purines, pyrimidines, vitamins and several amino acids
- Generally detected in association with rich, carbohydrate-containing sources
 - Some LAB are specialists
 - Adapted to few niches. Examples:
 - *Lactobacillus helveticus* (milk/whiskey malt) (Homoferm)
 - *Lactococcus cremoris* (milk) (Homoferm)
 - *Streptococcus thermophilus* (milk) (Homoferm)
 - *Pauicilactobacillus oligofermentans* (meat spoilage) (Heteroferm)
 - Ferments few sugars specific for the environment
 - Some LAB are generalists
 - Found in many environments. Examples:
 - *Lactiplantibacillus plantarum*
 - *Lacticaseibacillus casei*
 - *Lactococcus lactis*
 - Ferments many sugars

Generalist specialist Yu, A. O., Leveau, J. H., & Marco, M. L. (2020). Abundance, diversity and plant-specific adaptations of plant-associated lactic acid bacteria. *Environmental Microbiology Reports*, 12(1), 16-29.

Growth media (examples)

- General enumeration **of LAB**: de Man, Rogosa, Sharpe (MRS)

11. MRS MEDIUM

Casein peptone, tryptic digest	10.00	g	Trypticase soy broth	30.00	g
Meat extract	10.00	g	Yeast extract	3.00	g
Yeast extract	5.00	g	Agar, if necessary	15.00	g
Glucose	20.00	g	Distilled water	1000.00	ml
Tween 80	1.00	g	Adjust pH to 7.0 - 7.2.		
K ₂ HPO ₄	2.00	g			
Na-acetate	5.00	g			
(NH ₄) ₃ citrate	2.00	g			
MgSO ₄ x 7 H ₂ O	0.20	g			
MnSO ₄ x H ₂ O	0.05	g			
Distilled water	1000.00	ml			

Adjust pH to 6.2 - 6.5.

- General enumeration (not selective)

92: TRYPTICASE SOY YEAST EXTRACT MEDIUM

Trypticase soy broth	30.00	g
Yeast extract	3.00	g
Agar, if necessary	15.00	g
Distilled water	1000.00	ml

Adjust pH to 7.0 - 7.2.

- M17 for *Lactococcus* and *Streptococcus*

449: M17 MEDIUM FOR LACTIC STREPTOCOCCI

Casein peptone	5.00	g
Soy peptone	5.00	g
Bacto peptone	5.00	g
Yeast extract	2.50	g
Ascorbic acid	0.50	g
MgSO ₄ x 7 H ₂ O	0.25	g
Sodium beta-glycerophosphate	19.00	g
Lactose	5.00	g
Distilled water	1000.00	ml

1. Adjust pH to 6.9 ± 0.2.

2. Lactose should be sterilized by filtration.

- Media with modifications to improve growth/selectivity for specific bacteria
 - modified MRS (mMRS) with additional carbohydrates found in sourdough
- Also addition of salt, antibiotics, modification of pH or incubation temperature

638: LACTOBACILLUS MEDIUM III

Tryptone	10.00	g
Meat extract	5.00	g
Yeast extract	5.00	g
Glucose	7.00	g
Fructose	7.00	g
Maltose	7.00	g
Na-gluconate	2.00	g
Na-acetate x 3 H ₂ O	5.00	g
(NH ₄) ₂ citrate	2.00	g
K ₂ HPO ₄ x 3 H ₂ O	2.60	g
MgSO ₄ x 7 H ₂ O	0.10	g
MnSO ₄ x 4 H ₂ O	0.05	g
L-Cysteine HCl x H ₂ O	0.50	g
Tween 80	1.00	ml
Distilled water	1000.00	ml

Adjust pH to 6.3

Common Genera of LAB & differential growth

TABLE 1.1 Common Genera of LAB and Their Differential Characteristics

FAMILY	GENERA	SHAPE	CHARACTERISTICS								TYPE OF LACTIC ACID
			CO ₂ FROM GLUCOSE	GROWTH AT 10°C	GROWTH AT 45°C	GROWTH IN 6.5% NaCl	GROWTH IN 18% NaCl	GROWTH AT pH 4.4	GROWTH AT pH 9.6		
Aerococcaceae	<i>Aerococcus</i>	Cocci (tetrads)	-	+	-	+	-	-	-	+	L
Carnobacteriaceae	<i>Carnobacterium</i>	Rods	Variable ^a	+	-	ND	-	ND	-	-	L
Enterococcaceae	<i>Enterococcus</i>	Cocci	-	+	+	+	-	+	+	+	L
	<i>Tetragenococcus</i>	Cocci (tetrads)	-	+	-	+	+	Variable	+	L	
	<i>Vagococcus</i>	Cocci	-	+	-	-	-	+	Variable	ND	
Lactobacillaceae	<i>Lactobacillus</i>	Rods	Variable	Variable	Variable	Variable	-	Variable	-	D, L, DL	
(including the former Leuconostocaceae)	<i>Pediococcus</i>	Cocci (tetrads)	-	Variable	Variable	Variable	-	+	-	L, DL	
	<i>Leuconostoc</i>	Cocci ^b	+	+	-	Variable	-	Variable	-	D	
	<i>Fructobacillus</i>	Rods	+	±	-	±	-	Variable	±	D	
	<i>Oenococcus</i>	Cocci	+	+	-	Variable	-	Variable	-	D	
	<i>Weissella</i>	Rods/cocci	+	+	-	Variable	-	Variable	-	D, DL	
	<i>Periweissella</i>										
Streptococcaceae	<i>Lactococcus^c</i>	Cocci	-	+	-	-	-	Variable	-	L	
	<i>Streptococcus</i>	Cocci	-	-	Variable	-	-	-	-	L	

Note: ND, not determined.

^a When present, CO₂ production from glucose by Carnobacteria is weak.

^b Some *Weissella* strains and *Periweissella* are rod shaped.

^c In older literature, Lactococci are referred to as Group N streptococci.

Differential growth of LAB on carbohydrates

TABLE 84. Key characteristics of Group A lactobacilli (obligately homofermentative)^a

Species	<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>L. delbrueckii</i> subsp. <i>lactis</i>	<i>L. delbrueckii</i> subsp. <i>indicus</i>	<i>L. acidophilus</i>	<i>L. amylophilus</i>	<i>L. amylophilus</i>	<i>L. amylavorus</i>	<i>L. amylavorus</i> subsp. <i>avianarius</i>	<i>L. amylavorus</i> subsp. <i>araffinosis</i>	<i>L. crispatus</i>	<i>L. farciminis</i>	<i>L. gallinarum</i>	<i>L. gasseri</i>	<i>L. helveticus</i>
Phylogenetic group	de	de	de	de	de	de	de	de	sl	sl	de	u	de	de	de
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp	Lys-D-Asp
G+C content (mol%)	49-51	49-51	49-51	49-51	34-37	39	44-46	40.3	39-43	41.3	35-38	34-36	36-37	33-35	37
Lactic acid isomer(s)	d	d	d	d	dl	dl	l	dl	l(d)	dl	l(d)	dl	dl	dl	dl
Growth (°C) 15/45	-/+	-/+	-/+	-/+	-/+	-/+	+/-	-/+	-/ND	-/ND	-/+	+/-	+/+	-/+	-/+
<i>Carbohydrates fermented:</i>															
Amygdalin	-	-	+	-	+	d	-	+	d	d	+	+	+	+	-
Cellobiose	-	d	d	-	+	-	-	+	+	d	+	+	+	+	-
Galactose	-	-	d	-	+	+	+	+	d	-	+	+	+	+	+
Lactose	-	+	+	+	+	-	-	-	d	-	+	+	d	-	+
Maltose	d	-	+	-	+	+	+	+	+	+	+	+	+	d	d
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	d
Melibiose	-	-	-	-	d	d	-	-	d	-	-	-	+	d	-
Raffinose	-	-	-	-	d	d	-	-	+	-	-	-	+	d	-
Salicin	-	-	+	-	+	d	-	+	+	d	+	+	+	+	-
Sucrose	+	-	+	d	+	+	-	+	+	+	+	+	+	+	-
Trehalose	d	-	+	-	d	-	-	+	+	+	-	+	-	d	d

^aSymbols and abbreviations: +, 90% or more of strains are positive; -, 90% or more are negative; d, 11-89% of strains are positive; w, weak positive reaction; ND, no data available; (), isomers in parentheses indicate <15% of total lactic acid; mDpm, meso-diaminopimelic acid; de, *Lactobacillus delbrueckii*-group; sl, *Lactobacillus salivarius*-group; re, *Lactobacillus reuteri*-group; u, unique.

^bW. P. Hammes, unpublished results.

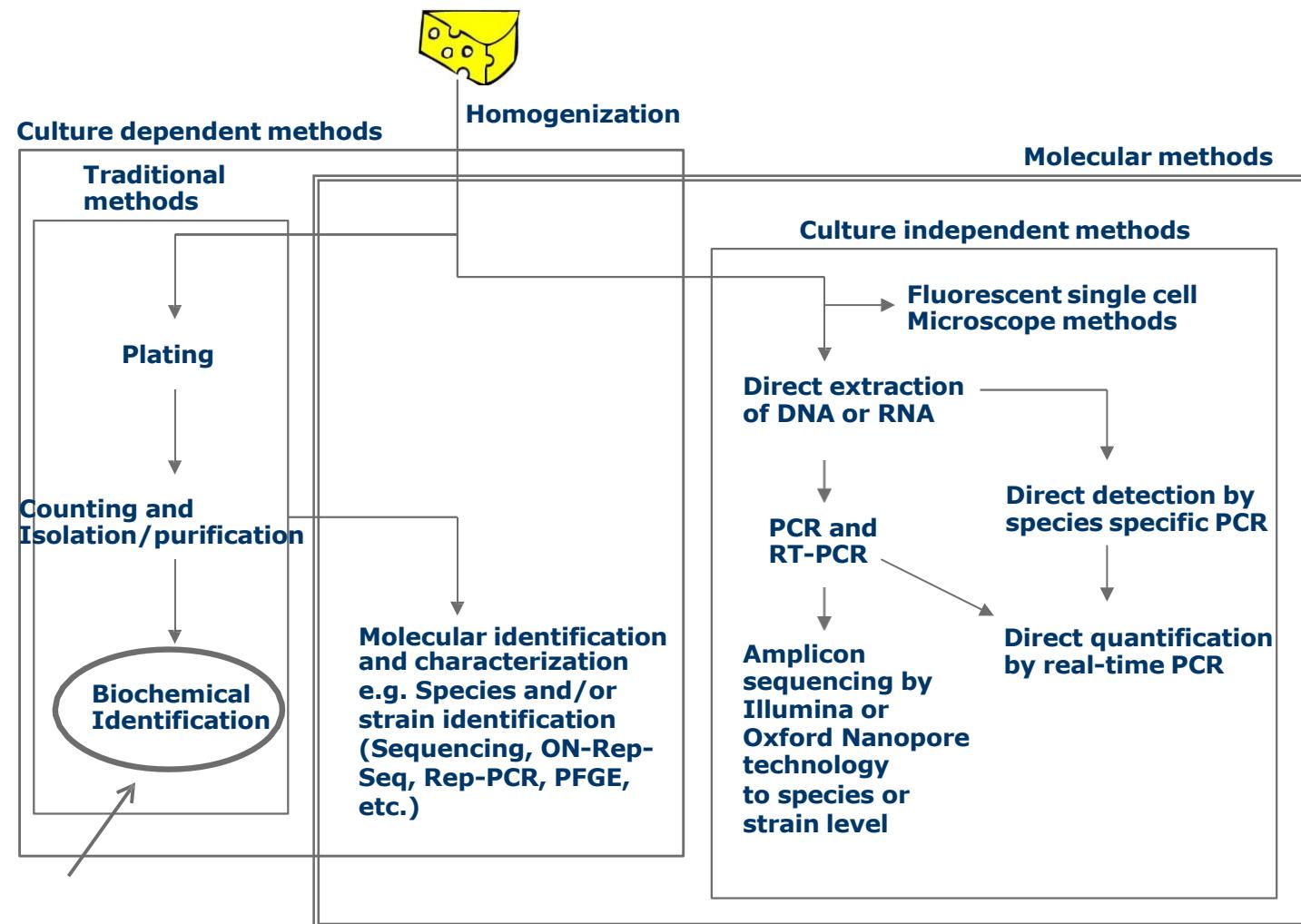
Useful test: API CHL 50 (Biomerieux)
Based on MRS+ one of 50 carbon sources



Further reading, free through KU – library (digital version):

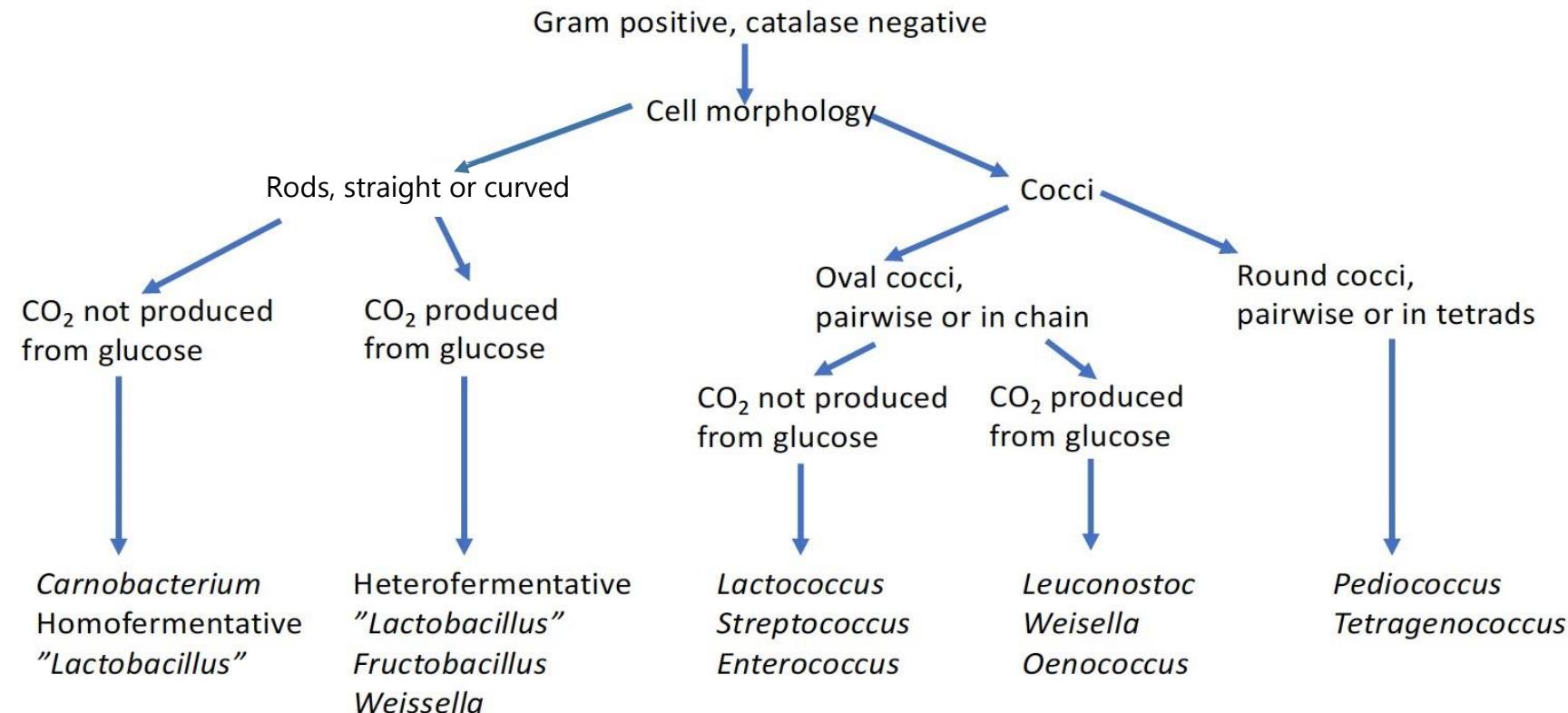
- The Prokaryotes (3rd Edition) Vol. 4
- Bergey's Manual Of Systematic Bacteriology (2nd Ed) Vol 3 – The Firmicutes

Detection of LAB from food



Modified from idea of Rantsiou & Cocolin (2006): Int. J. Food Microbiol. 108, 255-267

Simple scheme for phenotypic identification of LAB at genus level



Distinction of LAB from other gram positive bacteria often found in food and feed (genus level)

Cocci:

- *Staphylococcus* and *Micrococcus* are catalase positive (but a few LAB have catalase/pseudocatalase activity)

Rods:

- Aerobic sporeforming bacteria (e.g., *Bacillus* species) are catalase positive and produces spores
- *Listeria*, *Propionibacterium* and coryneform bacteria are catalase positive (but some LAB have catalase/pseudocatalase activity)
- *Clostridium* are catalase negative, but produces spores

Problems with morphology

Lb. casei →

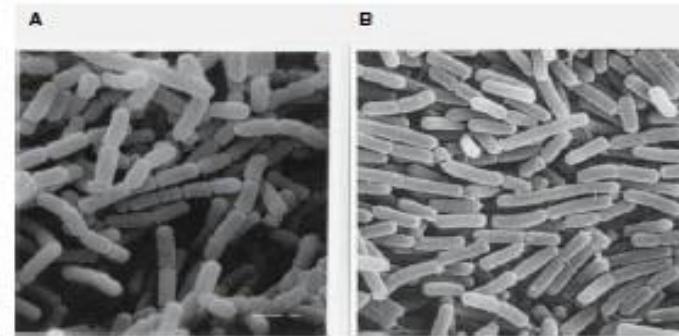


Fig. 10. Electron micrograph of A) *L. casei* and B) *L. acidophilus* (7000 ×; courtesy of Vittorio Bottazzi).

← *Lb. acidophilus*

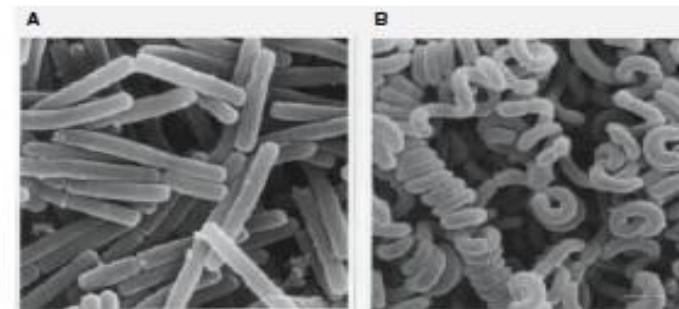


Fig. 11. Electron micrograph of *L. delbrueckii* subsp. *bulgaricus*. A) cells grown in MRS broth; and B) cells in spiral from MRS colonies (7000 ×; courtesy of Vittorio Bottazzi).

← ← *Lb. delbrueckii* subsp. *bulgaricus*

Phenotypic differentiation species level (within genus *Lactococcus*)

	Growth at 40°C	Growth at pH=9.2	Growth in 4 % NaCl	NH ₃ from Arginine	Acetoin Production	Citrate fermentation	Carbohydrates fermented						
							Galaktose	Lactose	Maltose	Ribose	Melibiose	Melitose	Raffinose
<i>Lc. lactis</i> subsp. <i>lactis</i>	+	+	+	+	-	-	+	+	+	+	-	-	-
<i>Lc. lactis</i> subsp. <i>cremoris</i>	-	-	-	-	-	-	+	+	-	-	-	-	-
<i>Lc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i>	+	+	d	d	+	+	+	+	+	d	-	-	-
<i>Lc. lactis</i> subsp. <i>hordniae</i>	-	ND	-	+	ND	ND	-	-	-	-	-	-	-
<i>Lc. garviae</i>	+	ND	+	+	ND	ND	+	+	d	+	d	-	-
<i>Lc. plantarum</i>	ND	ND	+	-	ND	ND	-	-	+	-	-	+	-
<i>Lc. raffinolactis</i>	+	+	+	d	-	-	+	+	+	d	+	d	+
<i>Lc. piscium</i>	-	ND	ND	-	ND	ND	+	+	+	+	+	+	+

The reactions in grey are extended Sherman reactions useful for identification of the *Lc. lactis* strains from starter cultures.

Lactococcus lactis biovar *diacetylactis* is a citrate fermenting variant that carry a plasmid that codes for a gene that is responsible for transport citrate into the cell. In addition, it has a citrate lyase gene on the chromosome.

Both genes are necessary for utilizing citrate to produce diacetyl and acetoin

Diacetyl = important flavor in cheese and butter

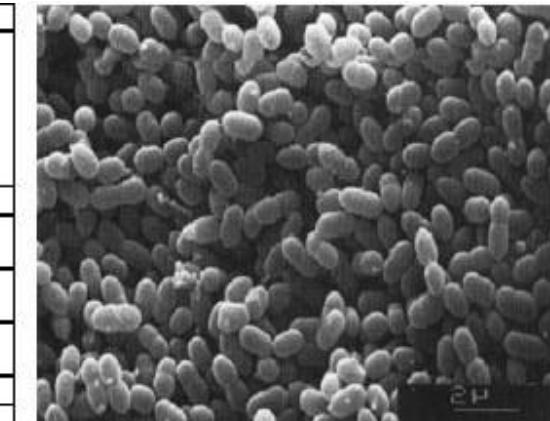
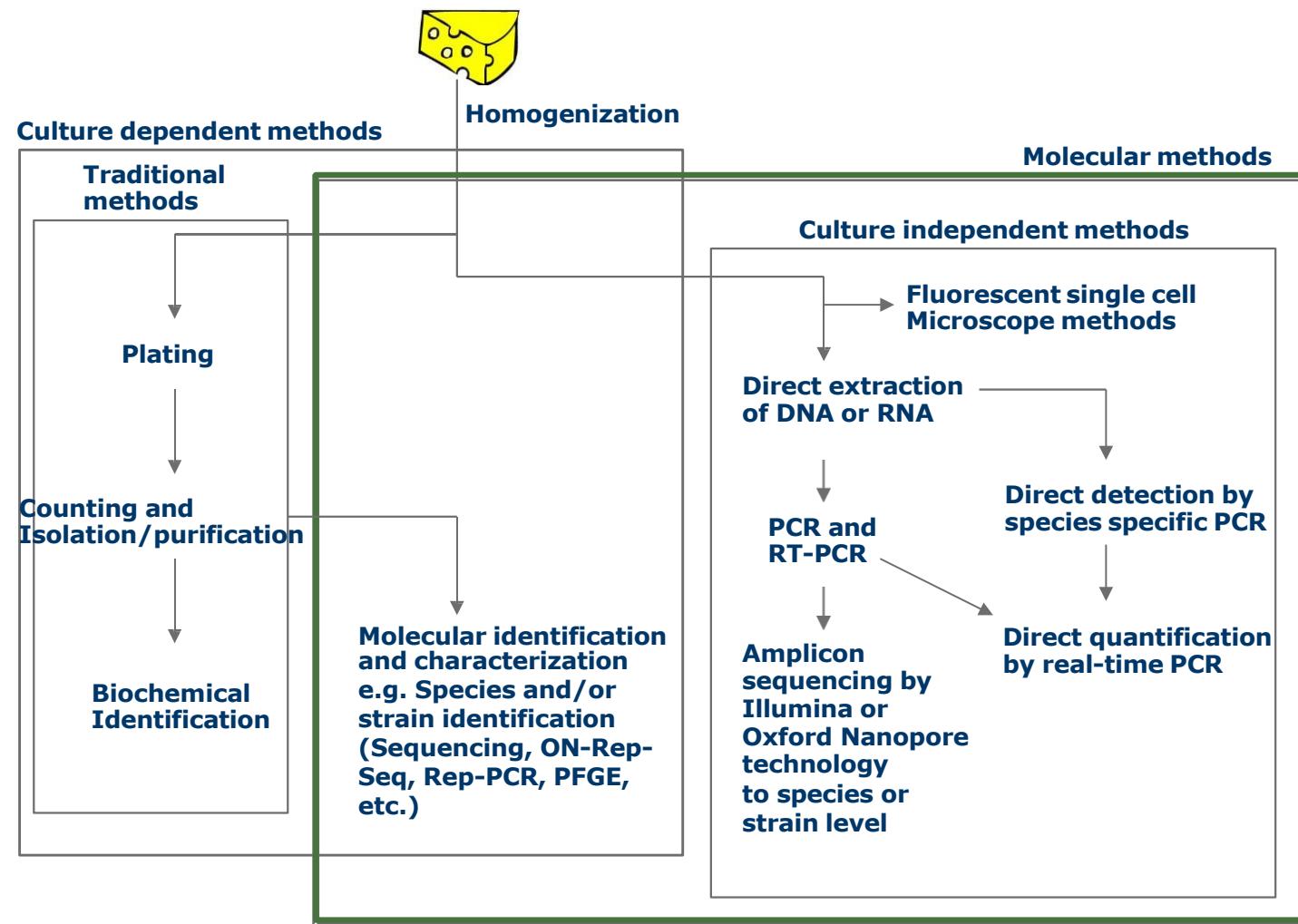


Fig. 5. Scanning electron micrograph of *Lactococcus lactis* subsp. "diacetylactis" growing in pairs of ovoid cells. Bar = 2 μ m.

Phenotypic characterisation is useful, but often incomplete/misleading

Detection of LAB from food



Modified from idea of Rantsiou & Cocolin (2006): Int. J. Food Microbiol. 108, 255-267

Molecular identification of LAB (species)

- PCR amplify 16S rRNA gene (both culture dependent and independent)
 - Sequence V1-V3 region (first approx. 500 bp of 1500 bp gene)
 - Make BLAST search in GenBank, Ribosomal Database or EzBioCloud.net
 - In many cases you will get a reasonable identification at species level



Commonly commercially available service through either Sanger or Illumina sequencing

Lactococcus tree based on 16S rRNA gene

- Less than 1% difference in 16S rRNA between *Lc. lactis* and *Lc. cremoris*
- **Cannot be discriminated**

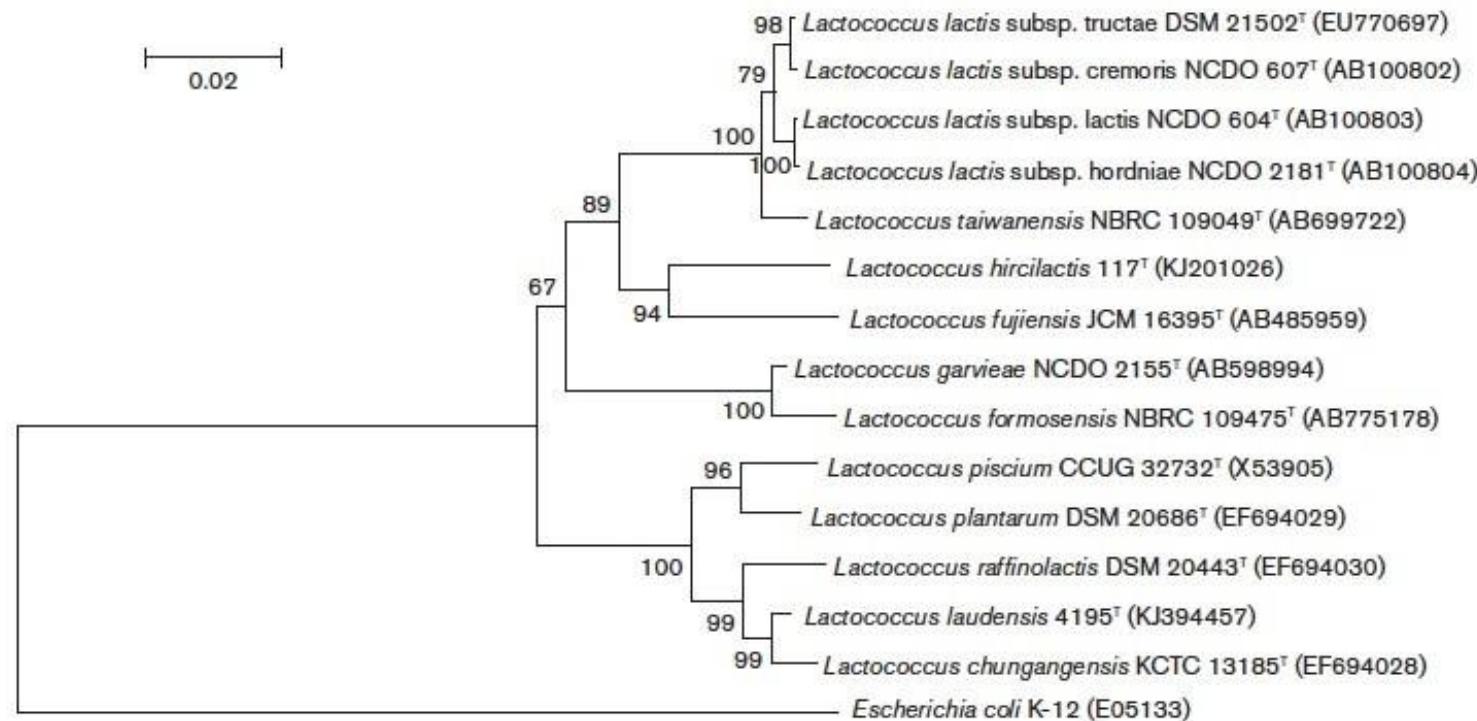


Fig. 1. Neighbour-joining tree with strains 117^T and 4195^T and other related lactococci, based on 16S rRNA gene sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. GenBank accession numbers are given in parentheses. *Escherichia coli* K-12 was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Molecular identification of LAB (species)

- PCR amplify 16S rRNA gene (both culture dependent and independent)
 - Sequence V1-V3 region (first approx. 500 bp of 1500 bp gene)
 - Make BLAST search in GenBank, Ribosomal Database or EzBioCloud.net
 - In many cases you will get a reasonable identification at species level
- PCR amplify one, two or more genes and sequence
 - *atpA*
 - *rpoA*
 - *pheS*
 - *recA*
 - *groEL*
 - Blast search in GenBank
- ON-Rep-Seq
- Whole genome sequencing (**it is the new gold-standard**)

Differentiation of *Lactococcus* – whole genome sequencing (WGS)

Table 1. ANI and dDDH values (%) between the type strains of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *tructae* and phylogenetically related reference strains

Strain	<i>L. lactis</i> subsp. <i>lactis</i> ATCC 19435 ^T (FMTF01000000)	<i>L. lactis</i> subsp. <i>hordniae</i> CCUG 32210 ^T (VXKC01000000)		<i>L. lactis</i> subsp. <i>cremoris</i> ATCC 19257 ^T (JXJZ01000000)		<i>L. lactis</i> subsp. <i>tructae</i> DSM 21502 ^T (JXKC01000000)		
	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)
<i>L. lactis</i> subsp. <i>lactis</i> ATCC 19435 ^T (FMTF01000000)	100	100						
<i>L. lactis</i> subsp. <i>hordniae</i> CCUG 32210 ^T (VXKC01000000)	97.6	79.9	100	100				
<i>L. lactis</i> subsp. <i>cremoris</i> ATCC 19257 ^T (JXJZ01000000)	86.9	32.5	86.4	31.5	100	100		
<i>L. lactis</i> subsp. <i>tructae</i> DSM 21502 ^T (JXKC01000000)	86.5	31.7	86.5	31.6	98.0	84.2	100	100
<i>L. taiwanensis</i> NBRC 109049 ^T (BNDT01000000)	79.1	23.7	79.0	23.7	77.7	22.0	77.7	21.9
<i>L. kimchii</i> S-13 ^T (SDAK01000000)	77.9	23.1	77.8	22.4	77.8	22.8	77.3	22.4
<i>L. allomyrinae</i> 1JSPPR-7 ^T (CP032627)	76.0	22.3	76.0	22.6	76.0	23.7	75.8	22.5
<i>L. protaetiae</i> KACC 19320 ^T (CP041356)	76.2	22.5	76.3	23.1	76.1	22.9	76.2	22.7
<i>L. hircilactis</i> DSM 28960 ^T (WITJ01000000)	73.4	24.6	72.4	21.4	73.0	23.5	72.7	23.0
<i>L. fujimensis</i> JCM 16395 ^T (JXJU01000000)	72.6	23.0	72.4	22.2	72.5	21.7	72.2	20.9

ANI:

Average Nucleotide Identity

ANI ≥95 = same species

dDDH

DNA-DNA hybridization

Differentiation of *Lactococcus* – whole genome sequencing (WGS)

ANI:
Average Nucleotide Identity

ANI ≥95 = same species

dDDH
DNA-DNA hybridization

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	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)	ANI (%)	dDDH (%)
<i>L. lactis</i> subsp. <i>lactis</i> ATCC 19435 ^T (FMTF01000000)	100	100						
<i>L. lactis</i> subsp. <i>hordniae</i> CCUG 32210 ^T (VXKC01000000)	97.6	79.9	100	100				
<i>L. lactis</i> subsp. <i>cremoris</i> ATCC 19257 ^T (JXJZ01000000)	86.9	32.5	86.4	31.5	100	100		
INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY	TAXONOMIC DESCRIPTION		 MICROBIOLOGY SOCIETY	Li et al., <i>Int. J. Syst. Evol. Microbiol.</i> 2021;71:004727 DOI 10.1099/ijsem.0.004727	84.2	100	100	
					22.0	77.7	21.9	
					22.8	77.3	22.4	
					23.7	75.8	22.5	
					22.9	76.2	22.7	
					23.5	72.7	23.0	
					21.7	72.2	20.9	

Elevation of *Lactococcus lactis* subsp. *cremoris* to the species level as *Lactococcus cremoris* sp. nov. and transfer of *Lactococcus lactis* subsp. *tructae* to *Lactococcus cremoris* as *Lactococcus cremoris* subsp. *tructae* comb. nov.

Ting Ting Li^{1,2}, Wen Li Tian^{1,*} and Chun Tao Gu^{1,2,*}

LAB physiology

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LAB physiology and their role in fermentations

- LAB are involved in (lactic) acid fermentations
 - Obligate fermentative
 - Lactic acid major end product of fermentation
- What is the role of LAB in fermented products?
 - Production of acids (and other antimicrobial compounds) inhibits unwanted microflora
 - Production of flavour compounds
 - Textural changes
- Where do they come from?
 - Present in the raw material
 - Added as starter cultures
 - Help from fermentation conditions

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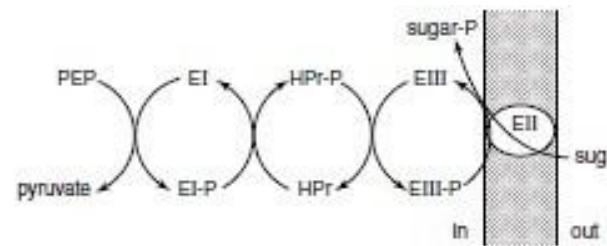
Production of acids

Uptake of available carbon sources into the cell

- Two main modes of uptake of carbohydrates

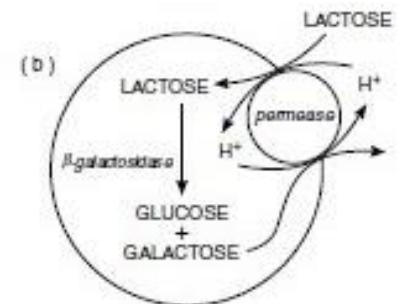
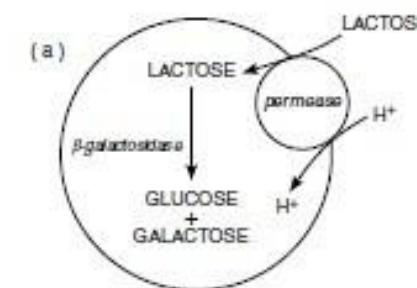
Sugar translocation through PEP:PTS

Sugar: Phosphoenolpyruvate: PhosphoTransfererase
Energy efficient as phosphorylation during uptake
makes transport "free"



Sugar permeases e.g.

- Sugar(Lactose)/H⁺ symport
- Lactose/galactose antiport



Example of lactose uptake:

PEP:PTS present in homofermentative LAB
for uptake of lactose

Present in *Leuconostoc*

Present in thermophilic bacteria:
S. thermophilus
Lb. delbrueckii subsp. *bulgaricus*

Uptake of carbohydrates (mono and di-saccharides)

PTS systems:

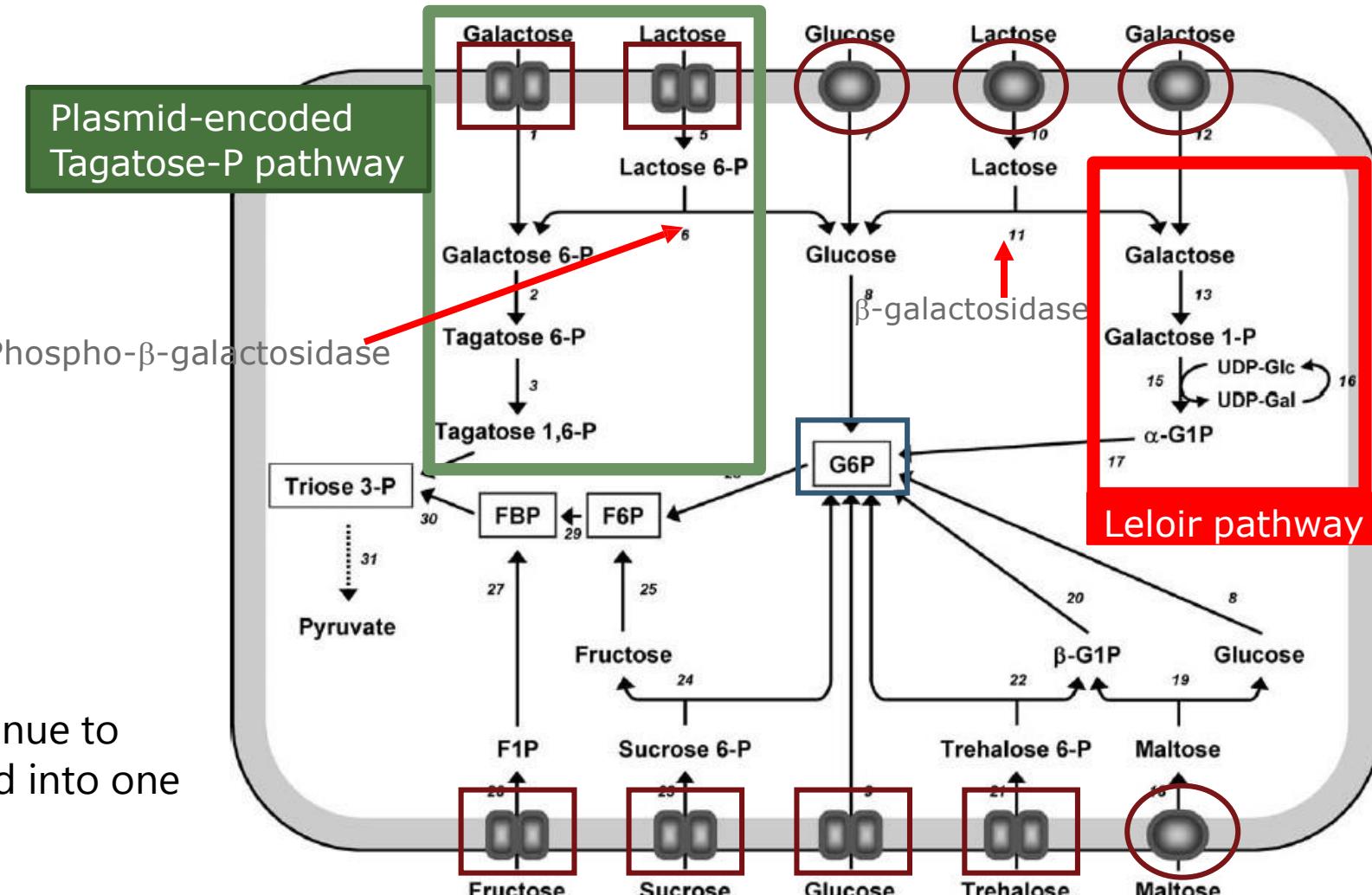


Permease systems:



Two fates of galactose:

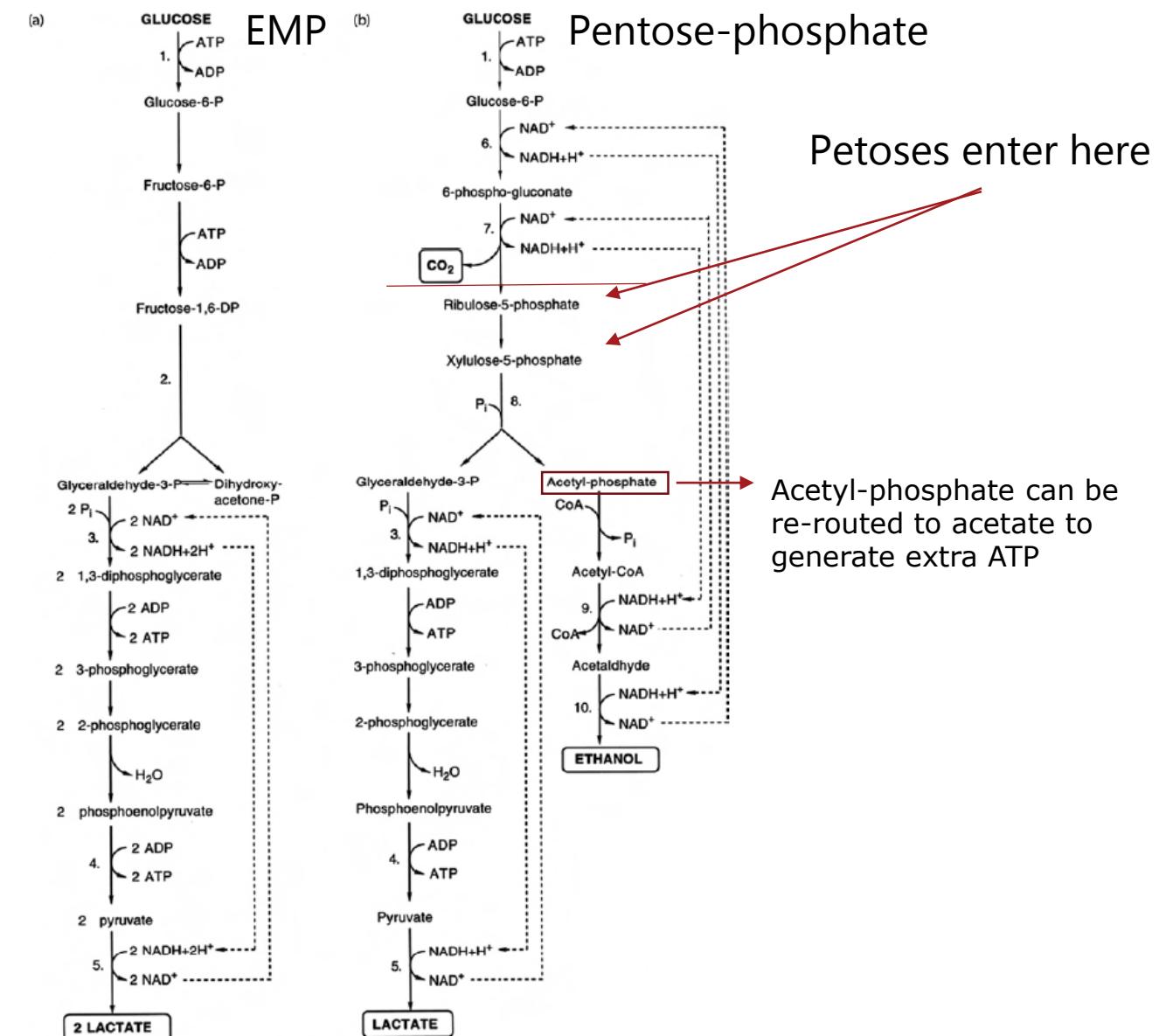
The others typically continue to glucose-6-phosphate and into one of the two pathways



Note: this is just a visual representation. Not all would be present in one species.

Homofermentative and heterofermentative pathways

- Division applies to fermentation of hexoses
- Some of the differences
 - Homo: net 2 ATP | Hetero: net 1 ATP
 - End products
 - Homo: lactate
 - Hetero: lactate, ethanol and CO₂
- Fermentation of pentoses
 - As a rule follow hetero-fermentation (even in homofermentative bacteria)
 - No CO₂
- Former facultative heterofermentative used homofermentative pathway for hexoses and heterofermentative pathway for pentoses
- Exceptions to this rule led to change in classification



Homofermentative and heterofermentative pathways

Homofermentative

Lactococcus

Streptococcus

Pediococcus

Enterococcus

Re-classified Lactobacilli:

Lactobacillus (many important for dairy kept this name)

Amylolactobacillus (starch fermenting)

Holzapfelia (insect and flower associated)

Ligilactobacillus (host associated lifestyle, intestine)

Former facultative heterofermentative

Re-classified Lactobacilli:

Lacticaseibacillus (different food fermentation incl. dairy)

Latilactobacillus (different fermented products, e.g. meat, cheese, plant)

Dellaglioa (meat spoilage)

Liquorilactobacillus (fermented plant material and liquid beverages)

Lactiplantibacillus (different fermented foods, incl. dairy, plant, cereals)

Bombilactobacillus (from bees or bumble bees)

Campanilactobacillus (meat, spor drought, plant, dairy products)

Lapidilactobacillus (spirit cellar walls, cabbage and beer fermentations, meat spoilage)

Agrilactobacillus (related to soil)

Scheiferilactobacillus (spoiled beverage fermentations, including dairy and plant fermentation)

Paralactobacillus (chilli fermentate)

Loigolactobacillus (spoilage)

Heterofermentative

Leuconostoc

Oenococcus

Weissella

Carnobacterium

Re-classified Lactobacilli:

Furfurilactobacillus (sourdough and spoiled beverage) fermentations)

Paucilactobacillus (from plant and meat fermentations)

Limosilactobacillus (intestine, plant fermentations, dairy fermentations)

Secundilactobacillus (occurs in secondary fermentations, or spoilage)

Levilactobacillus (different food fermentations and intestine)

Fructilactobacillus (flowers, sourdough, insect intestine)

Acidilactobacillus (acid tolerant, isolated from vinegar)

Apilactobacillus (associated with bees and spoiled beverages)

Lentilactobacillus (slow growing, associated with food fermentations, many

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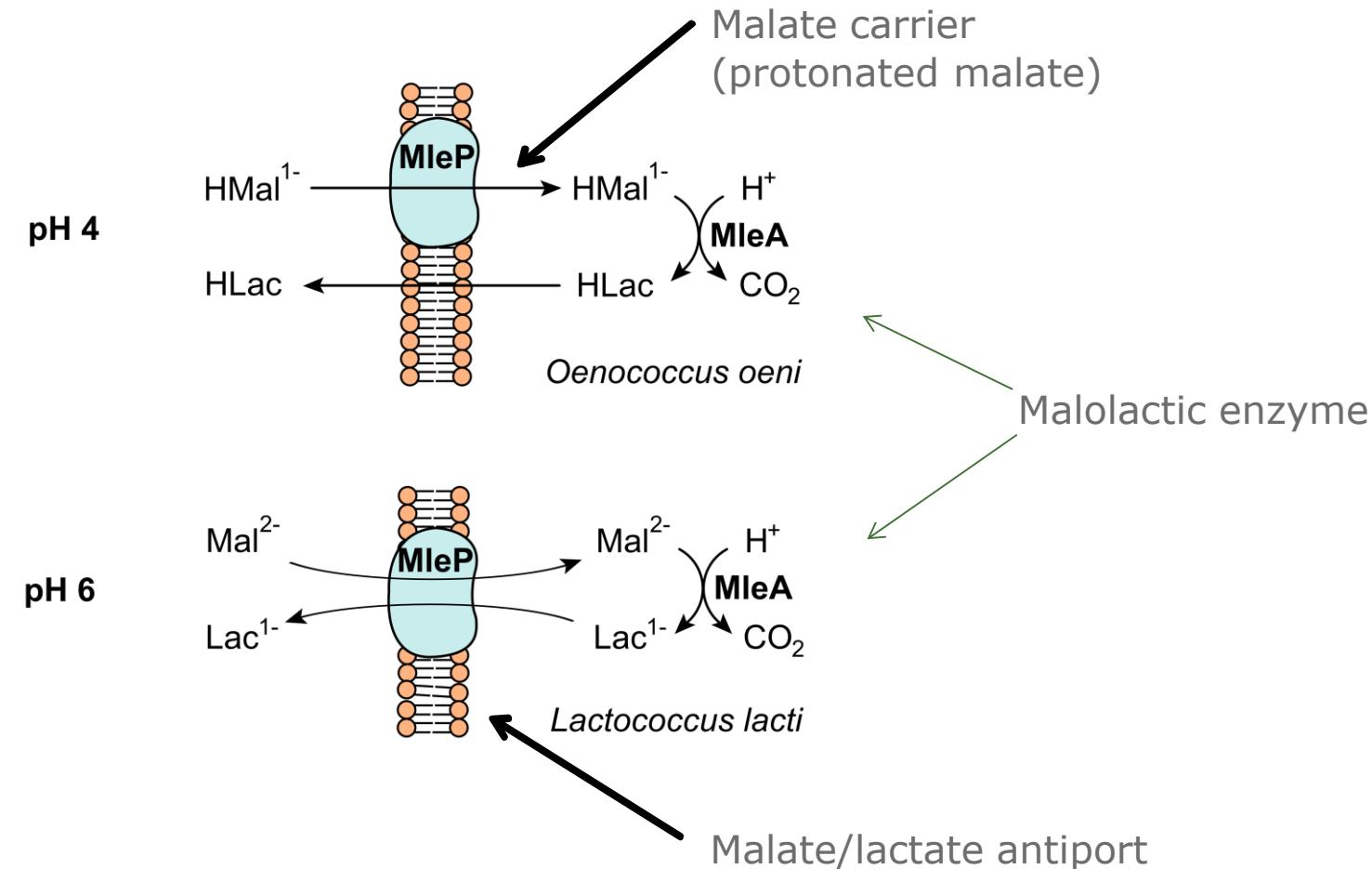
Production of flavour compounds

- Conversion of organic acids
 - Malolactic fermentation
 - Citrate fermentation
- Proteolysis and amino-acid catabolism

Malolactic fermentation

- Malic acid is abundant in grapes and gives wines a tart taste
- Particularly pronounced in red wines but also white and sparkling wines
- Malolactic fermentation is a process of removal of malic acid by LAB
 - *Oenococcus oeni*
 - Some *Pediococcus*
 - Some *Lactobacillus*
 - Performed either after alcoholic fermentation or concurrently
- LAB additionally stabilise wine by removing remaining fermentable carbohydrates
→ Contribute to enhanced aroma and flavor complexity
- If not controlled it can have negative effect

Disclaimer: Technically not a fermentation but the enzymatic decarboxylation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid

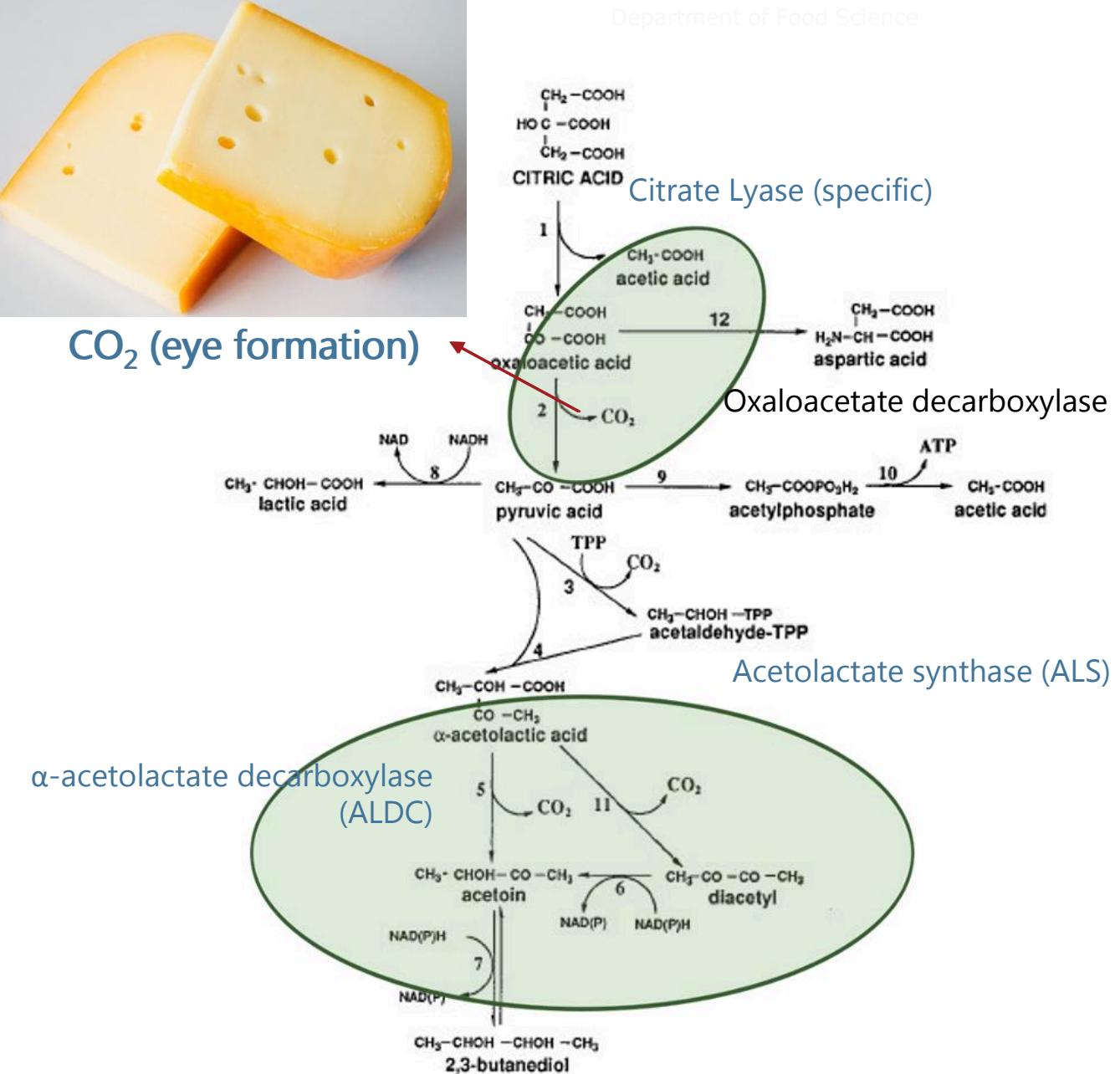


Citrate fermentation

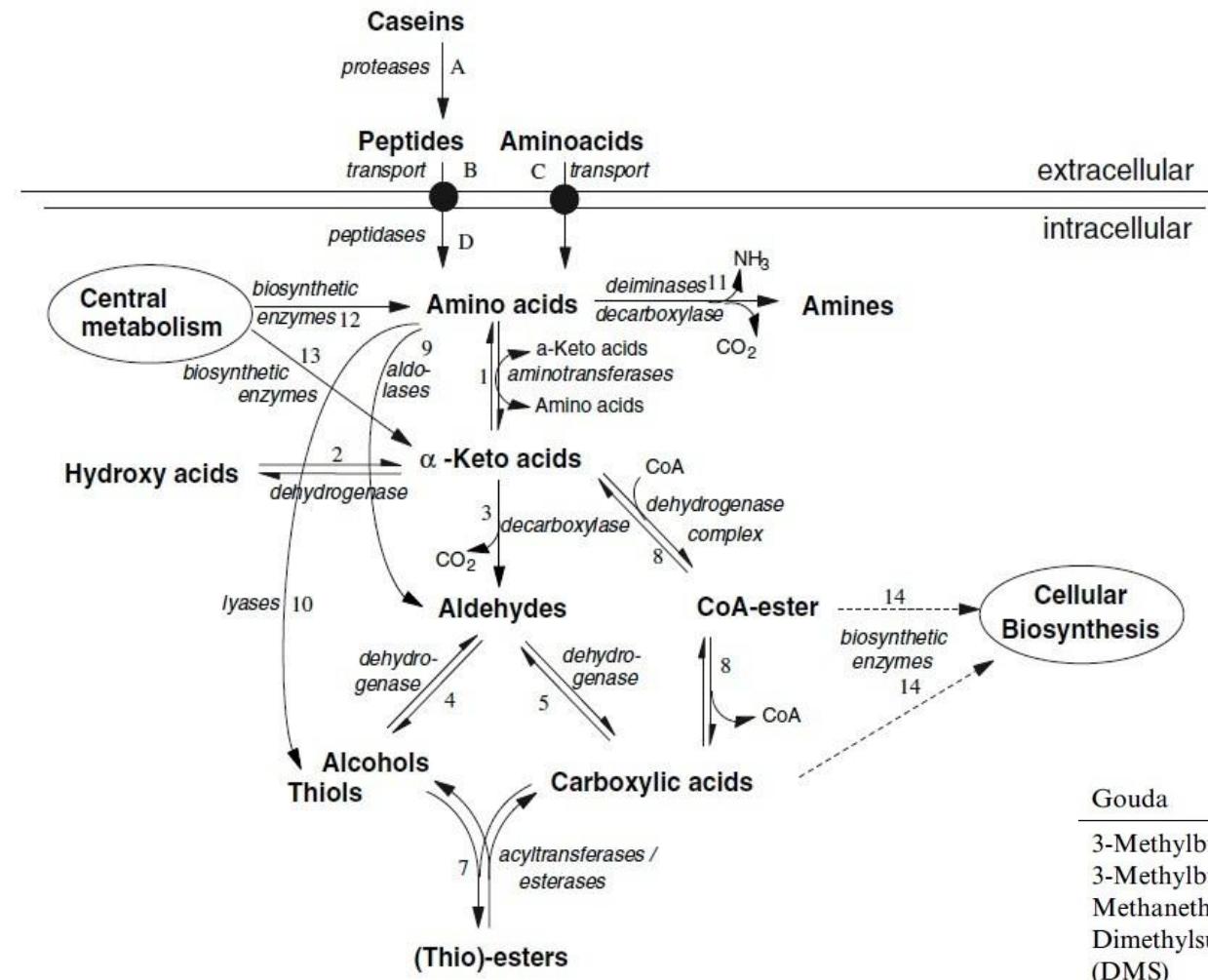
- Citrate can be fermented only by a limited number of lactic acid bacteria (Cit^+).
 - *Lactococcus lactis* biovar. *diacetylactis* (or $\text{Cit}^+ L. Lactis$) and *Leuconostoc*
- Its degradation usually results in the formation of unusual fermentation products such as diacetyl, acetoin, butanediol and acetaldehyde.
- Concurrent production of CO_2 results in eye formation in cheeses such as Gouda or Danbo (but not Swiss-type)
- The formation of the buttery aroma compound diacetyl is seen as positive in dairy products such as butter (milk) and cottage cheese, but it is detrimental in products such as beer, fermented sausage and wine.



CO_2 (eye formation)



Proteolysis and amino acid catabolism



Amino acid metabolism is very complex, and will be further described in the course "Dairy Microbiology"

Examples of flavour compounds from amino-acid catabolism in cheeses

Gouda	Cheddar	Camembert	Swiss-type (and Maasdam)
3-Methylbutanal	3-Methylbutanal	3-Methylbutyrate	Methional
3-Methylbutanol	Isovaleric acid	3-Methylbutanal	3-Methylbutanal
Methanethiol	Methional	Methional	Skatole
Dimethylsulphide (DMS)	Methanethiol	Methanethiol	
2-Methylpropanol	DMDS	DMS	
Dimethyltrisulphide (DMTS)	DMTS	Benzaldehyde	
		Phenylacetaldehyde	

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Textural changes

- Acid production
 - In dairy products pH drop leads to denaturation of milk proteins (casein) and formation of gel structure (e.g. sour cream with *Lactococcus* and *Leuconostoc* or yoghurt with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*)
- Gas (CO_2) production
 - Previously mentioned eye formation in cheeses (citrate fermentation by *L. lactis* biovar. *diacetylactis*, *Leuconostoc*)
- Antimicrobial compounds
 - Organic acids, ethanol, diacetyl, bacteriocins
 - Indirectly affect texture by inhibiting growth of other bacteria, yeasts and moulds
 - E.g. delayed texture softening in kimchi by addition of *Leuconostoc mesenteroides*
- Exopolysaccharide (EPS) production
 - In dairy products increased thickness and improved mouthfeel of fermented milks e.g. långfil (*Lactococcus cremoris*) or low-fat yoghurt (*Streptococcus thermophilus*)
 - In sourdough (lactobacilli, *Leuconosotc*, *Weisella*) increased viscosity of the sourdough, higher specific volume, lower firmness and reduced retrogradation of starch

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Where do they come from?

- Present in the raw material
 - LAB are ubiquitous bacteria found in a very wide range of raw materials
 - Names of LAB often reflect their origin (e.g. *Lactiplantibacillus plantarum* -leaves, *Lentilactobacillus kefiri* -kefir, *Companilactobacillus kimchi* -kimchi, *Oenococcus oeni*- wine, *Lactococcus lactis* -milk, *Lactococcus cremoris* –cream, *Lactobacillus helveticus*- Switzerland, *Lactobacillus delbrueckii* subsp. *bulgaricus*- Bulgarian yoghurt)
 - Bacteria isolated from the raw material to be fermented will likely be adapted to this material
 - Ability to ferment available sugars, co-exist with other microbes (or inhibit them), determine tolerance to oxygen
- Added as starter cultures (covered in detail in Dairy Microbiology)
 - To ensure presence of the bacteria of interest
 - Added at amounts that ensure desired effect
 - Same composition at each batch helps control consistent quality
- Help from fermentation conditions
 - We manipulate conditions to favour our bacteria of interest
 - We heat-treat the raw material to kill off competition
 - Change fermentation temperature to the optimal for our bacterium
 - Add salt to levels inhibitory for others
 - Allow or limit access of oxygen depending on the bacterium/material

Taxonomy and the species concept Part 1

Tessa Canoy

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KØBENHAVNS UNIVERSITET



Content

1. Definitions taxonomy and phylogeny
2. Phylogeny
3. Microbial taxonomy: from domain to species
 - > Species concept in Bacteria
 - > Species concept in Yeasts and moulds
4. Subspecies, variants and strains

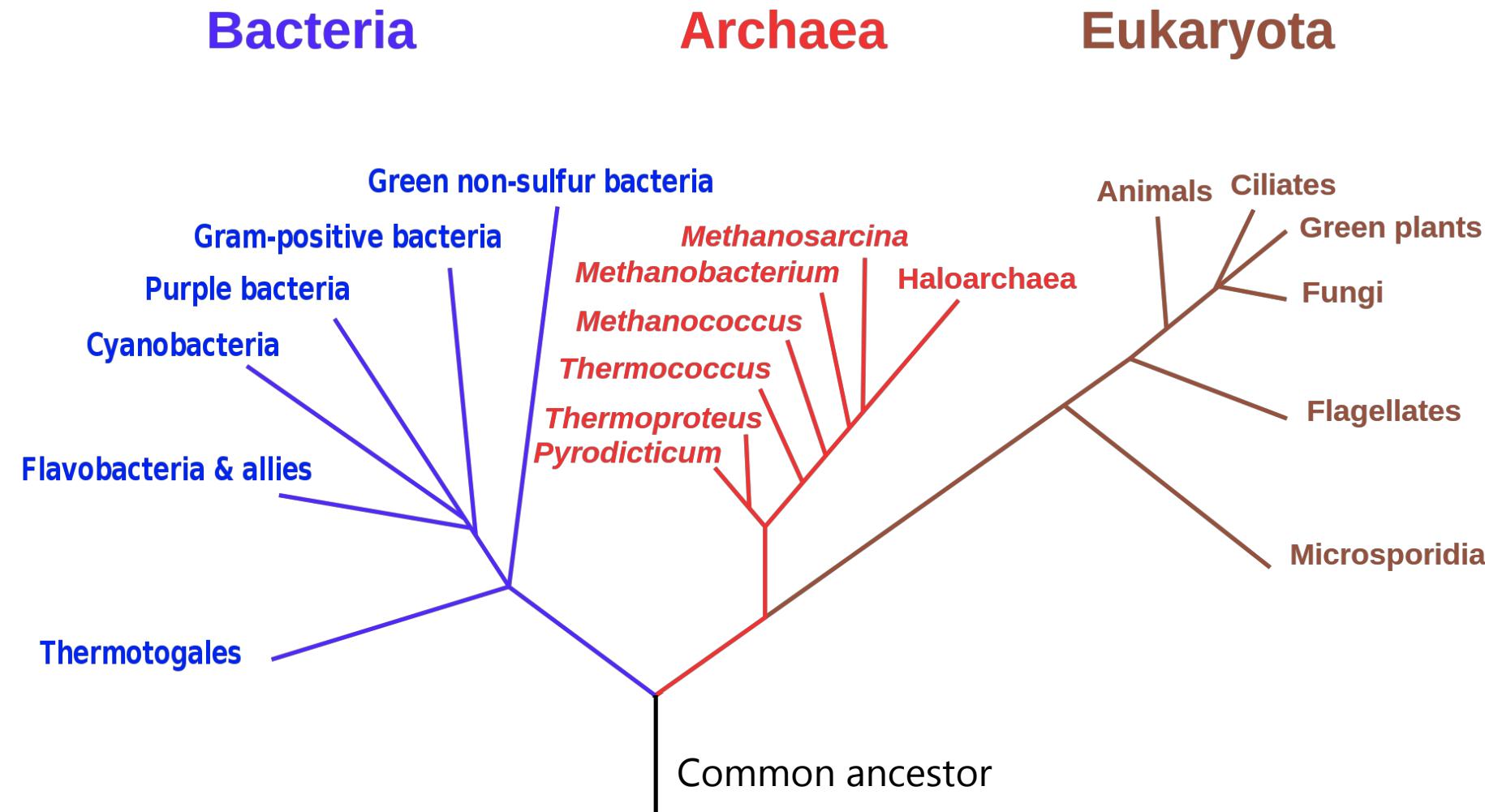
Literature

- Christensen, H., Olsen, J.E. (2018). Sequence-Based Classification and Identification of Prokaryotes. In: Christensen, H. (eds) Introduction to Bioinformatics in Microbiology. Learning Materials in Biosciences. Springer, Cham.

Supplementary

- Carro, L., Peix, Á., Velázquez, E. (2021). The Taxonomy of Bacteria in the Genomic Era. In: Villa, T.G., de Miguel Bouzas, T. (eds) Developmental Biology in Prokaryotes and Lower Eukaryotes. Springer, Cham.
- Hugenholtz, P., Chuvochina, M., Oren, A., Parks, D. H., & Soo, R. M. (2021). Prokaryotic taxonomy and nomenclature in the age of big sequence data. *the ISME Journal*, 15(7), 1879-1892.

1. Taxonomy and phylogeny: what is the difference?



Picture by Chiswick Chap

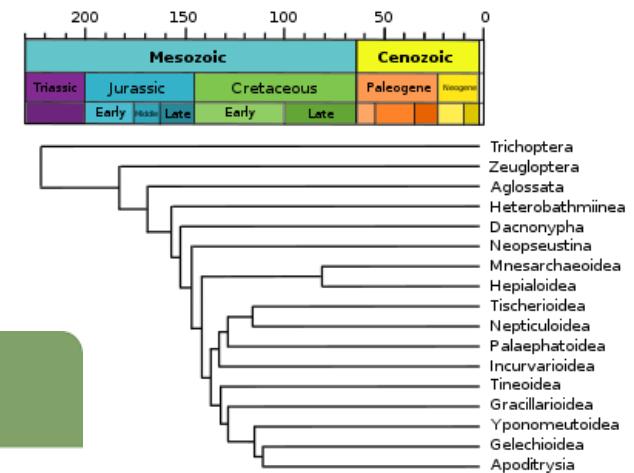
Definitions

Taxonomy: the naming and classification of organisms based on shared properties, can be based on:

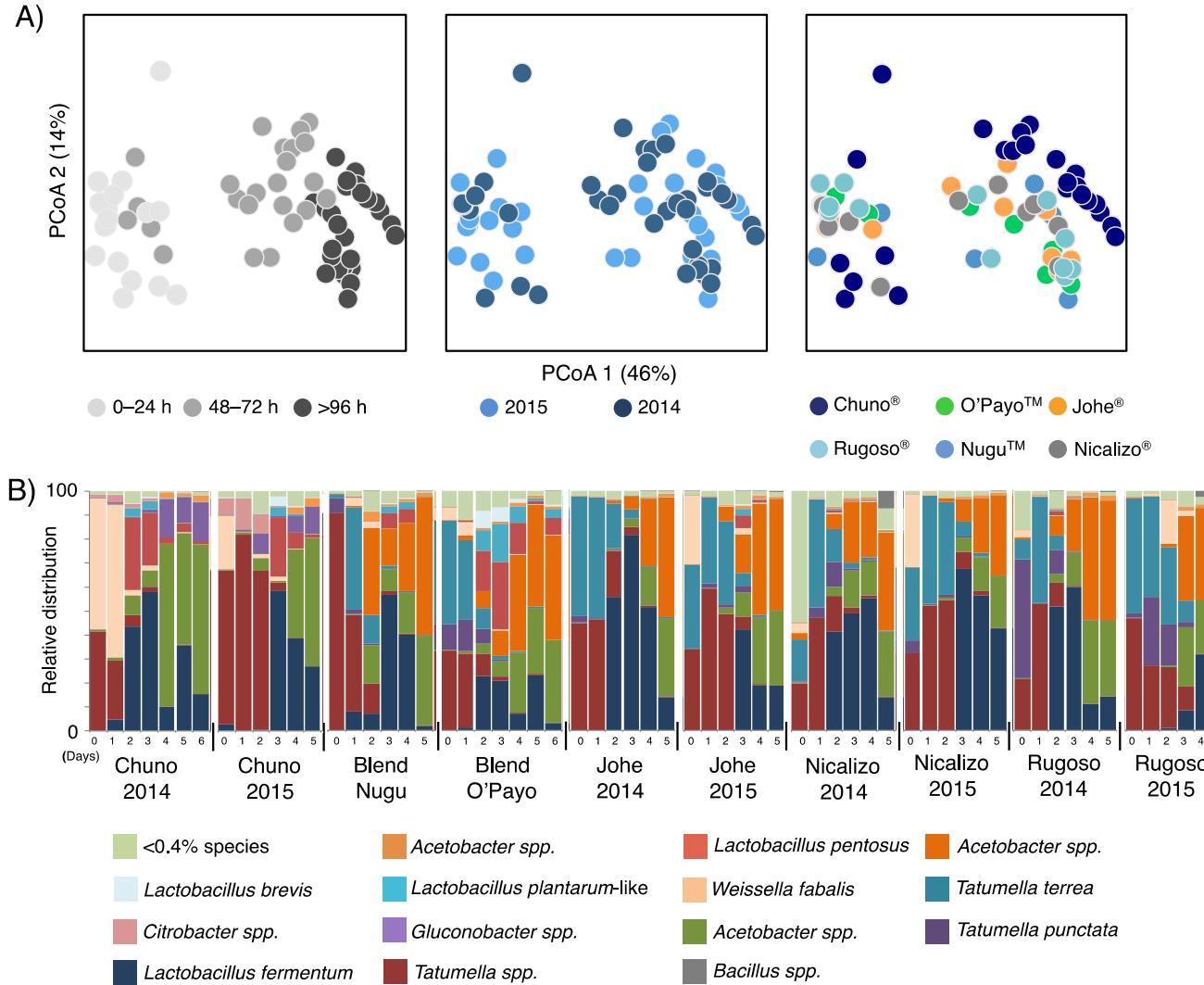
- (1) Genotype
- (2) Phenotype
- (3) Phylogeny** - The evolutionary development or history of a species or a taxonomic group of organisms

Nomenclature: the systematics of naming

HIERARCHY OF BIOLOGICAL CLASSIFICATION



Why do we need taxonomy and phylogeny?



Linking cocoa varietals and microbial diversity of Nicaraguan fine cocoa bean fermentations and their impact on final cocoa quality appreciation

Zoi Papalexandratou^{a,b}, Kristina Kaasik^c, Laureano Villagra Kauffmann^a, Albert Skorstengaard^c, Gregoire Bouillon^c, Julie Leth Espensen^{a,c}, Lars H. Hansen^e, Rasmus Riemer Jakobsen^c, Andreas Blennow^d, Lukasz Krych^c, Josué L. Castro-Mejía^c, Dennis Sandris Nielsen^{c,*}



To get meaning out of data like this

(no need to interpret the graphs for the course 😊)

Taxonomy and phylogeny in a broader context

Isolation



Sampling



Colony purification



DNA extraction

Identification

Identification methods



Microscopy
Micro/macro
morphologies

```
atccggcataatccgggtttgttccaaaggaa  
aaacgttgttcacggaaaggccagacggaaatttttt  
ccccggcggtgggttacggccaggacagtgcgtt  
ggggaaaaacccctcgccgtatggtgctgg  
ggccactcgcttaatgtatgtttcagg  
gactacacctacggtaacagtttct  
ggtgaaatttatcgatggcc
```

Sequencing

Taxonomy

Hello
my name is

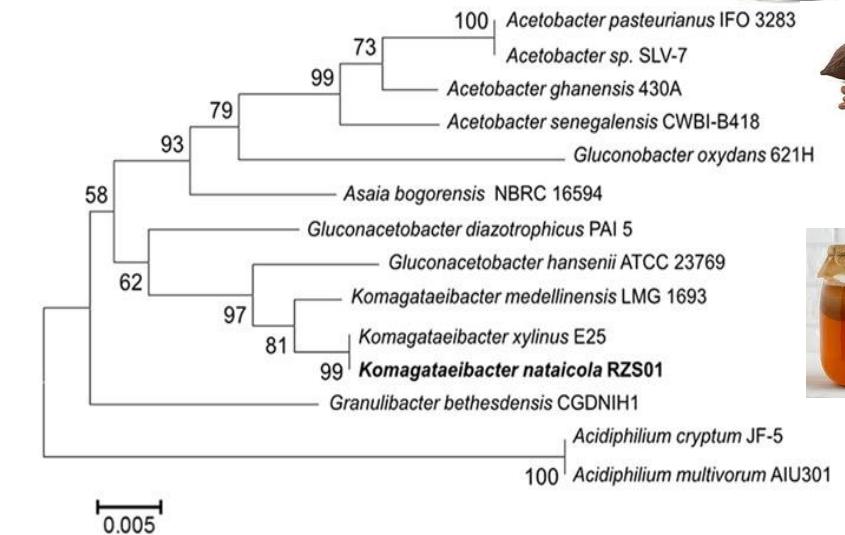
Bacillus subtilis

That's not my name

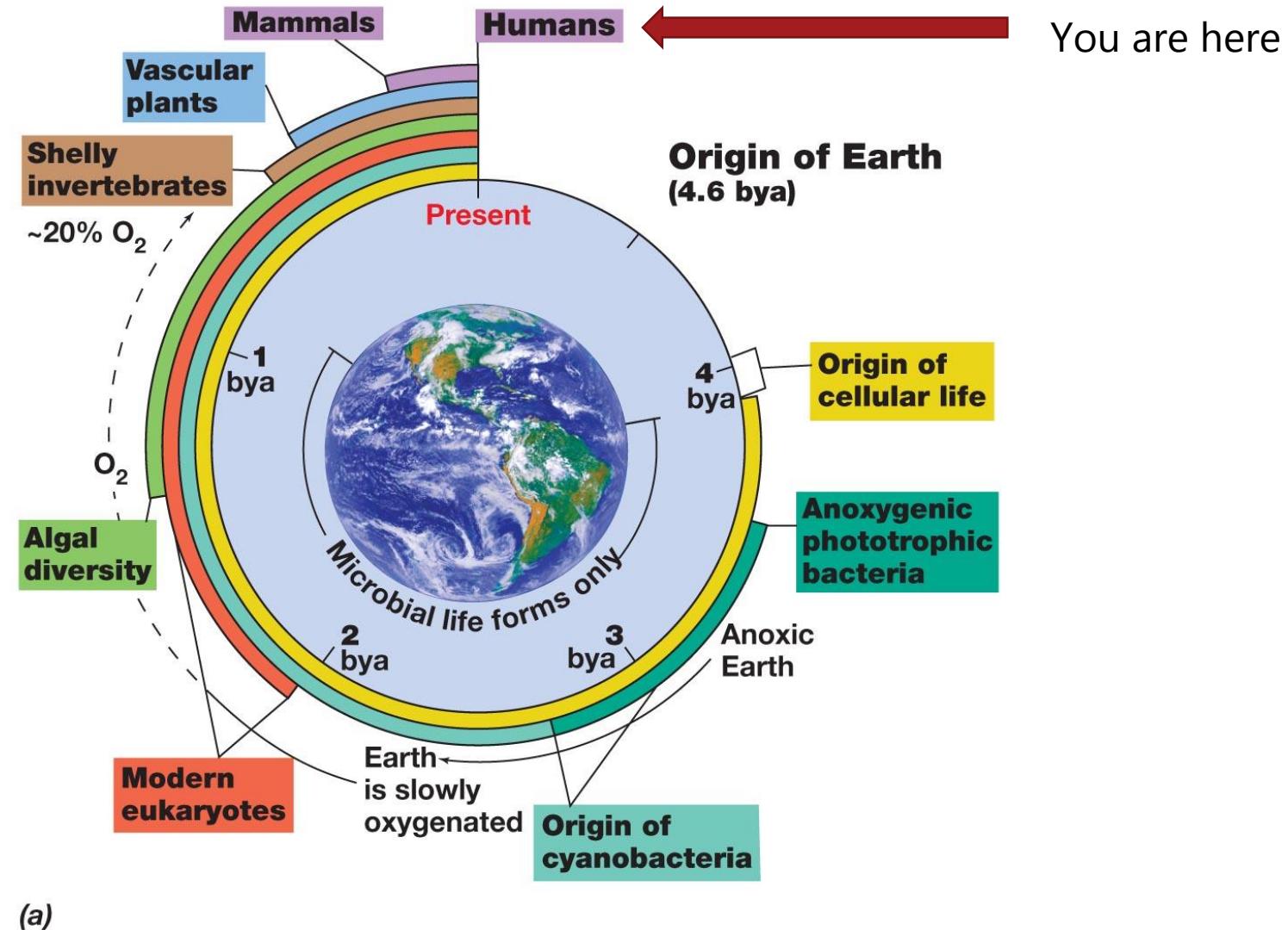
Bacillus cereus

Yeast taxonomy

Phylogeny



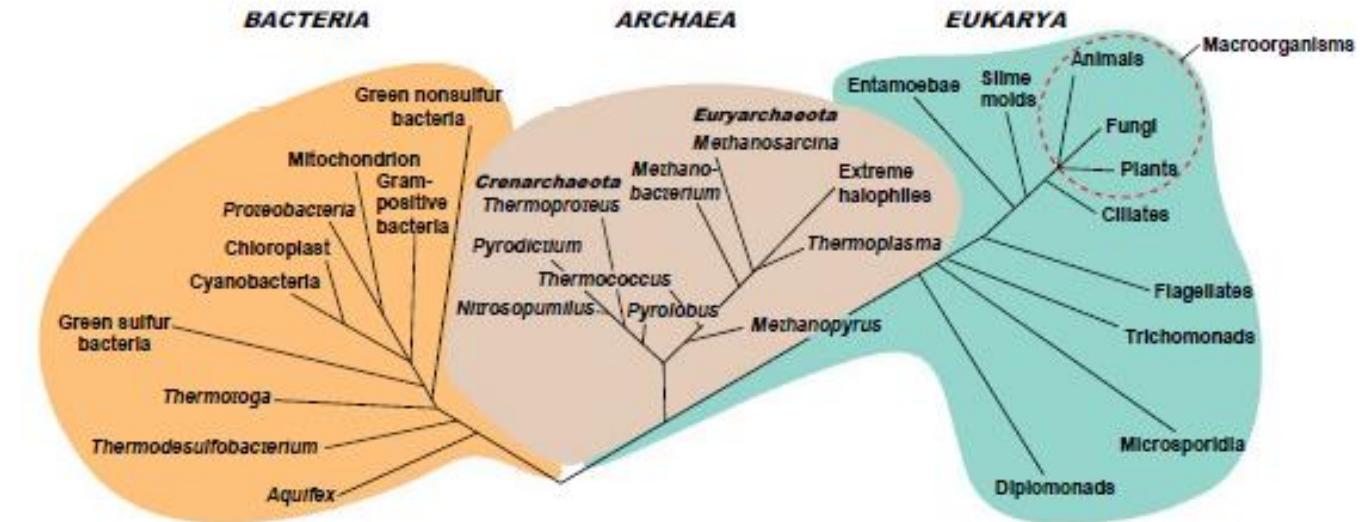
2. Phylogeny



Relatedness in food microbes

The three domains of life

- Eukarya
- Archaea (prokaryotes)
- Bacteria (prokaryotes)

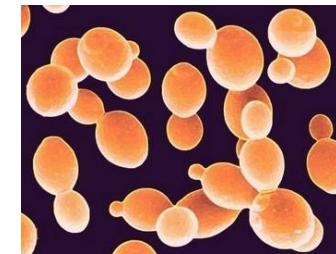


Who is most similar?



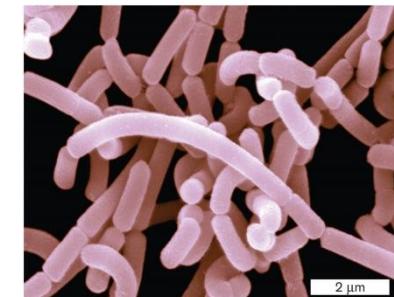
Homo sapiens

≈



Saccharomyces cerevisiae

≠



Lactobacillus acidophilus

© DENNIS KUNKEL/VISUALS UNLIMITED

Three domains of life can be explained by the ribosomes

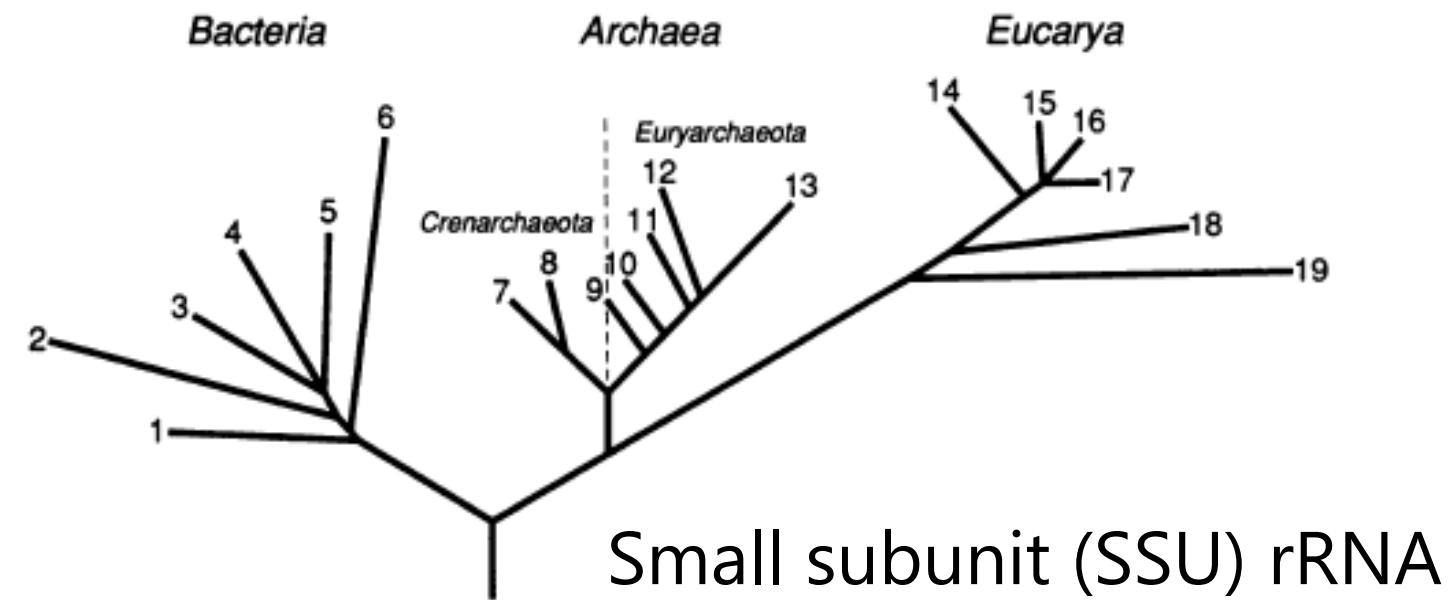
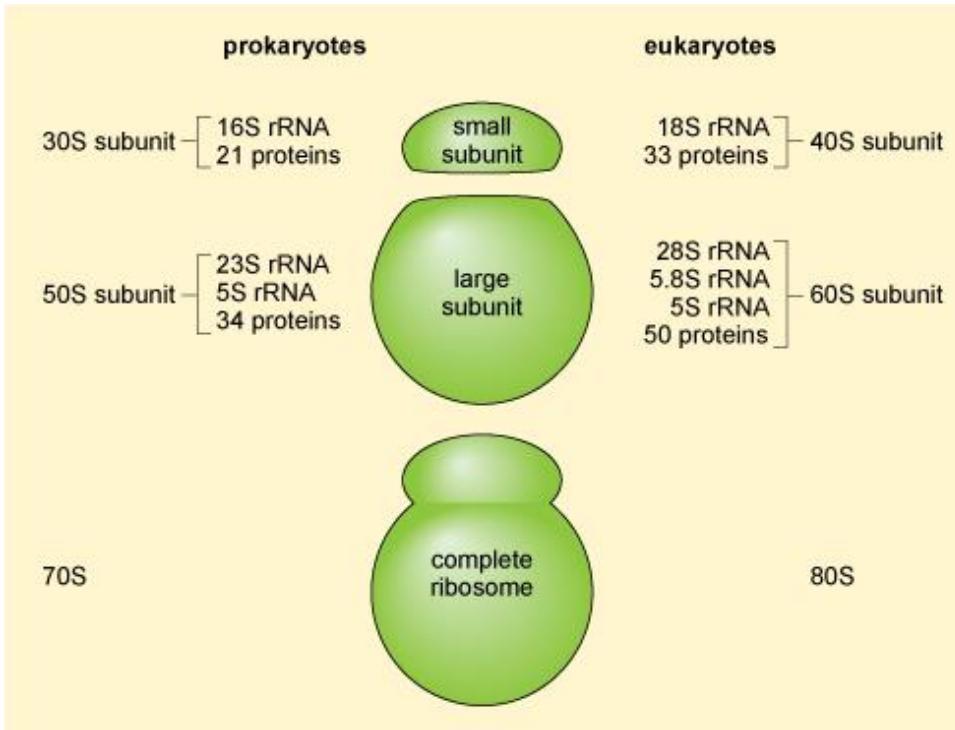


FIG. 1. Universal phylogenetic tree in rooted form, showing the three domains. Branching order and branch lengths are based upon rRNA sequence comparisons (and have been taken from figure 4 of ref. 2). The position of the root was determined by comparing (the few known sequences of pairs of paralogous genes that diverged from each other before the three primary lineages emerged from their common ancestral condition (27). [This rooting strategy (28) in effect uses the one set of (aboriginally duplicated) genes as an outgroup for the other.] The numbers on the branch tips correspond to the following groups of organisms (2). Bacteria: 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; 5, the Gram-positive bacteria; and 6, the green nonsulfur bacteria. Archaea: the kingdom Crenarchaeota: 7, the genus *Pyrodictium*; and 8, the genus *Thermoproteus*; and the kingdom Euryarchaeota: 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacterales; 12, the Methanomicrobiales; and 13, the extreme halophiles. Eucarya: 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the microsporidia.

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4576–4579, June 1990
Evolution

Towards a natural system of organisms: Proposal for the domains
Archaea, Bacteria, and Eucarya

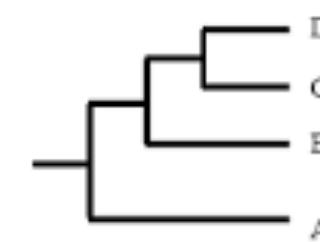
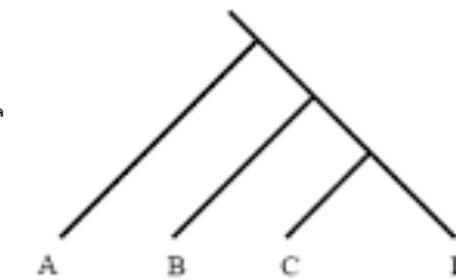
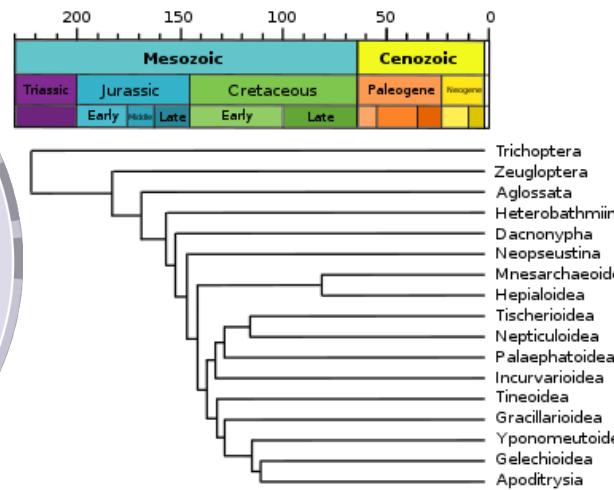
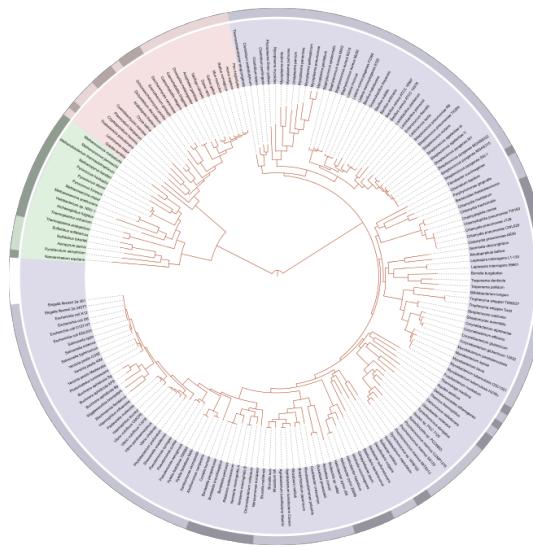
(Euryarchaeota/Crenarchaeota/kingdom/evolution)

CARL R. WOESE^{*†}, OTTO KANDLER[‡], AND MARK L. WHEELIS[§]

Phylogenetic trees

Phylogenetic trees depict evolutionary history

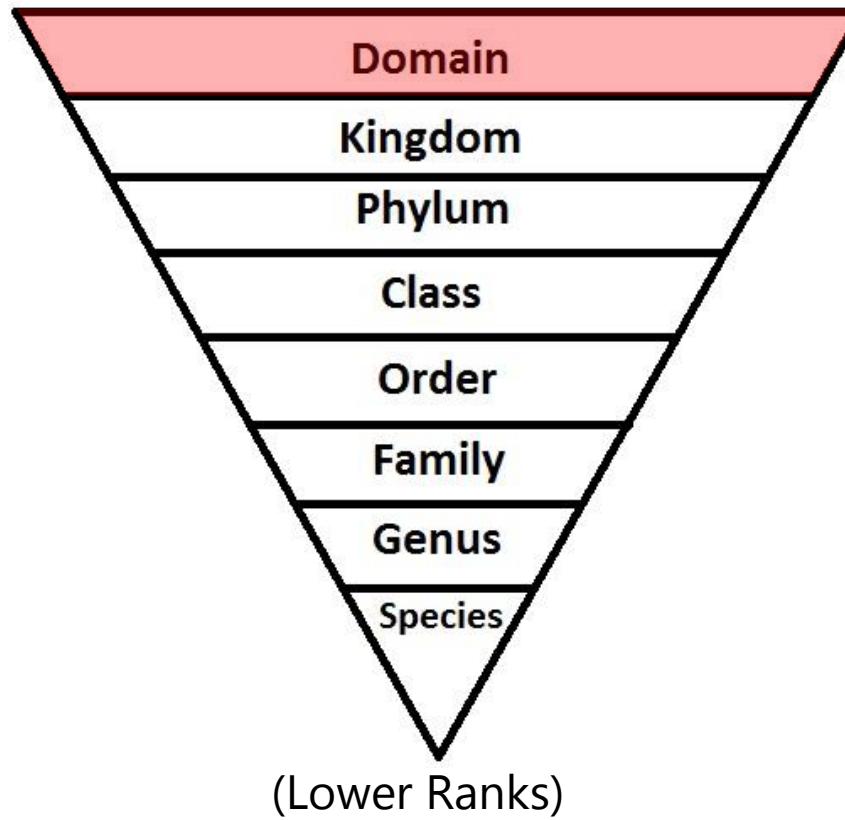
- Can be based on different molecular markers (DNA, RNA, protein)
- Comes in different types and visualization ‘formats’



- Can represent single genes, multiple genes, or regions (e.g. 16S)

3. Microbial taxonomy: from domain to species

(Higher Ranks)



- **Bacteria**
 - *None assigned*
 - Firmicutes
 - Bacilli
 - Lactobacillales
 - Lactobacillaceae
 - *Lactobacillus*
 - *L. acidophilus*
- **Eukarya**
 - Fungi
 - Ascomycota
 - Saccharomycetes
 - Saccharomycetales
 - Saccharomytaceae
 - *Saccharomyces*
 - *S. cerevisiae*

Domains of life - Prokaryotes vs. Eukaryotes

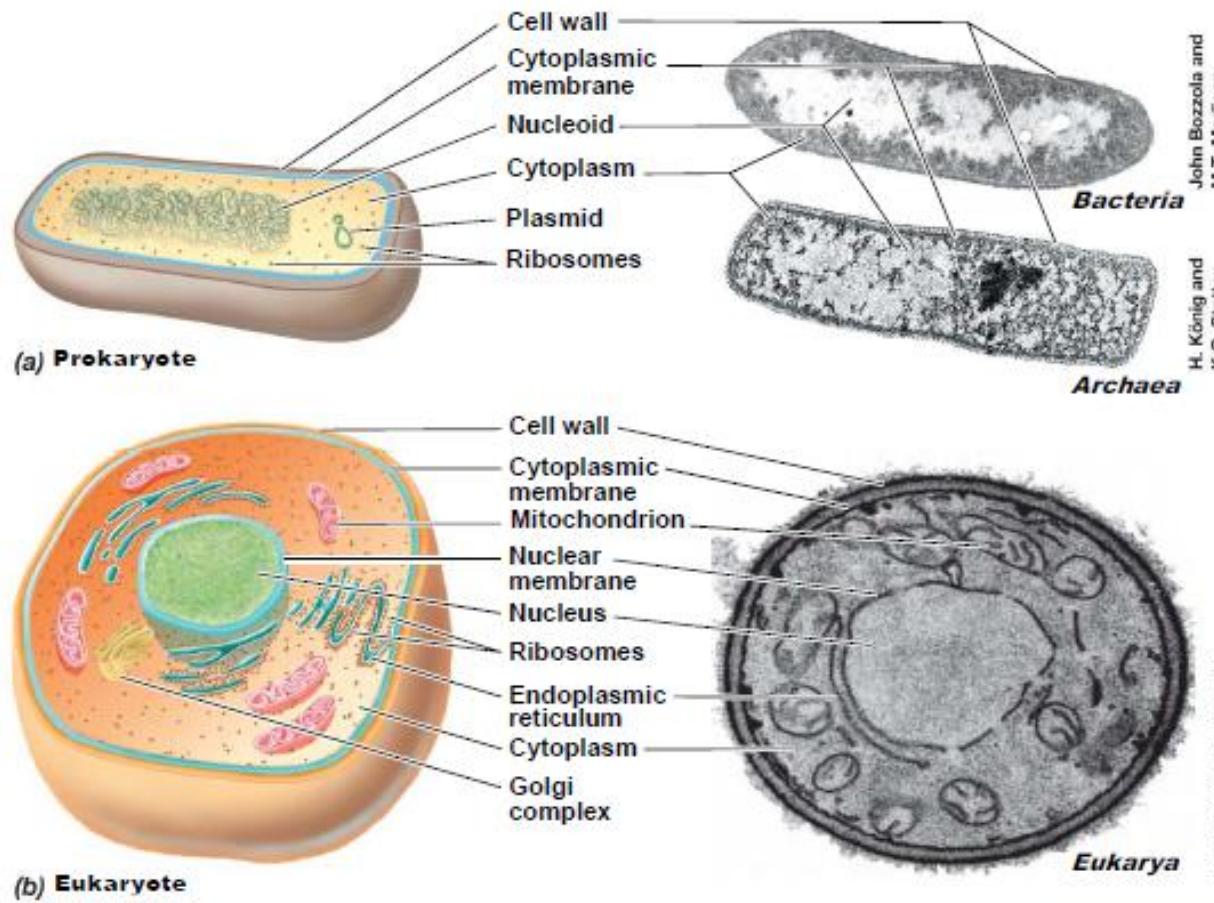


Figure 1.2

Two “fundamental” cell types:

- **Eukaryotes** (Greek, *eu* = true, *karyon* = kernel/nuclei)
- **Prokaryotes** (*pro* = before)

Many clear and evident differences

Two types of prokaryotes:

- **Bacteria**
- **Archaea**

Archaea and Bacteria look very much the same (but are very different)

Domains of life - Prokaryotes vs. Eukaryotes

	Prokaryotes	Eukaryotes
DNA	DNA is naked	DNA bound to protein
	DNA is circular	DNA is linear
	Usually no introns	Usually has introns
Organelles	No nucleus	Has a nucleus
	No membrane-bound	Membrane-bound
	70S ribosomes	80S ribosomes
Reproduction	Binary fission	Mitosis and meiosis
	Single chromosome (haploid)	Chromosomes paired (diploid or more)
Average Size	Smaller (~1–5 µm)	Larger (~10–100 µm)



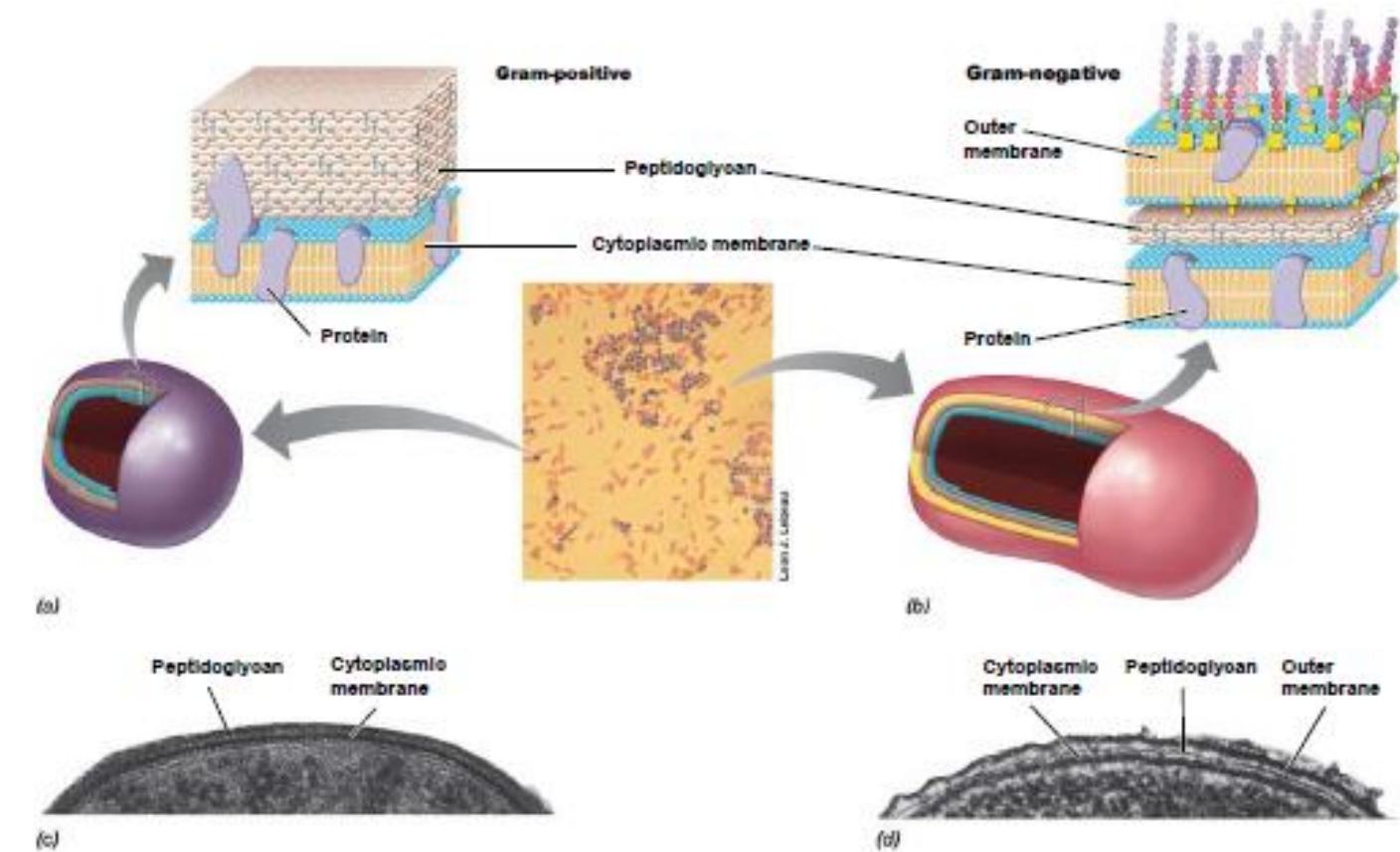
Prokaryotes: Gram positive and Gram negative bacteria

(Almost) all bacteria have a cell wall, protects against osmotic stress

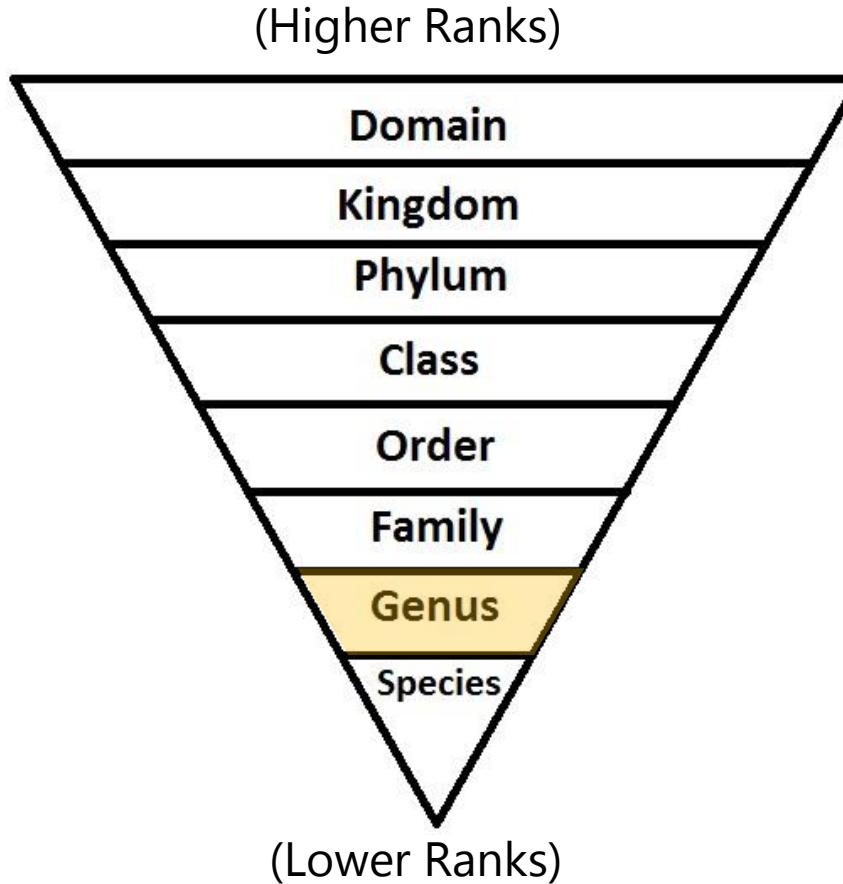
Bacteria can be divided in **Gram positive** and **Gram-negative** types

Gram+ (LAB + *Bacillus*)

Gram - (most acetic acid bacteria)



Microbial taxonomy: genus

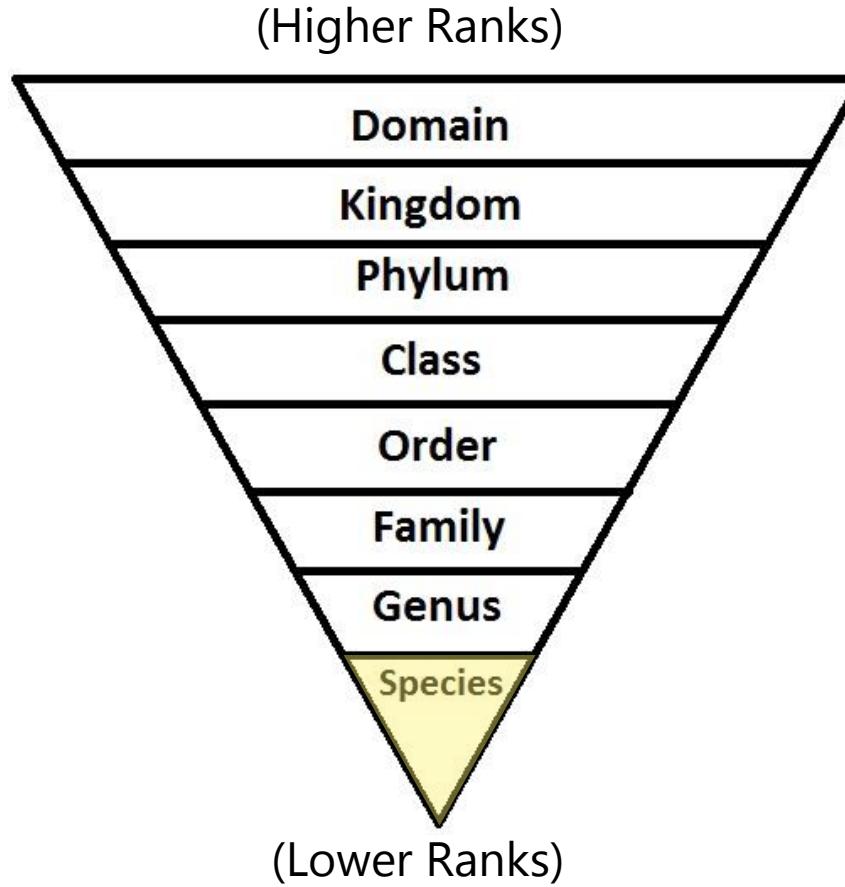


Genus definitions are less restrictive than for species definitions:

- **G+C content** varies less than 10 % within a genus
However, different genera can have the same GC content
- Groups of species showing phylogenetic and/or phenotypic similarity
Today new developments in classifications are based on 16S rDNA, 23S rDNA and conserved genes

New divisions and naming can be expected in future

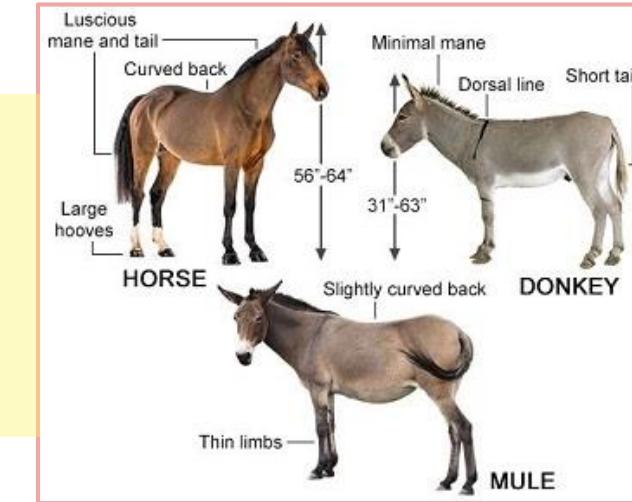
Microbial taxonomy: the species concept



How to define **species** for microbes?

In animals, sexual reproduction is an important characteristic to define a species

Male donkey & female horse → Mule



Can we use sexual reproduction to define species in bacteria and/or fungi?

Assignment (5 min):

How do we define species in
microorganisms?

Taxonomy and the species concept Part 2

Tessa Canoy

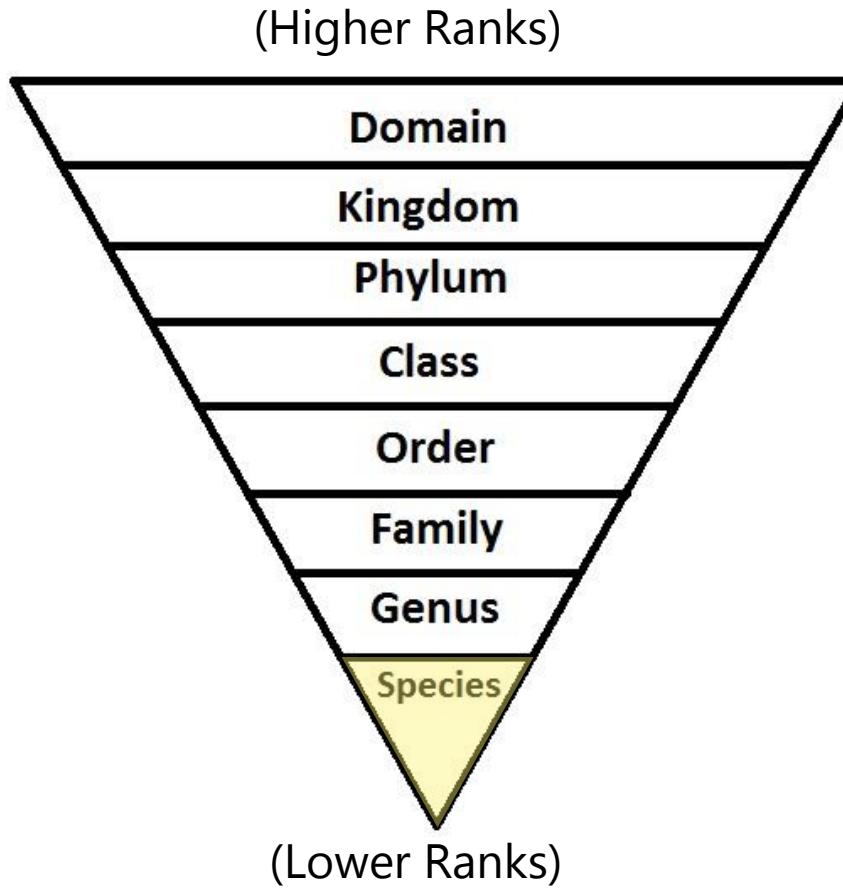
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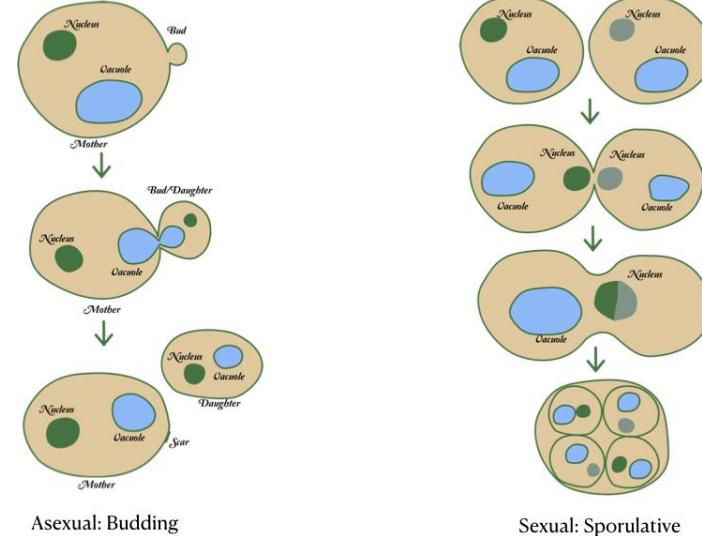
The species concept in microbiology



Bacteria: no sexual reproduction

Fungi: some fungi have sexual reproduction, but not all

Yeast Reproduction Methods



→ Sexual reproduction is not a good (only) measure to define microbial species, we need other methods

How do we differentiate **bacterial** species?

➤ DNA:DNA hybridisation (old fashion way)

Same species DDH >70%

➤ 16S rRNA gene sequencing

Same species 16S rRNA ≥97%-98.7%

➤ Whole genome sequencing

- Average Nucleotide Identify (ANI)

Same species ANI >95-96%

- Digital DNA-DNA hybridisation values (dDDH)

Same species dDDH >70%

16S rRNA sequences and whole genome sequencing could be combined for more dedicated taxonomy

How do we differentiate **bacterial** species?

- DNA:DNA hybridisation (old fashioned way)
 - **16S rRNA gene sequencing** ≥97%

≥97% same species (rule of thumb)
≈98.7% would be more accurate

16S rRNA (and hence also the 16S rRNA gene) contains a number of variable (9) and conserved regions

- The **variable regions** are highly variable in sequence between even closely related species
 - The **conserved regions** are on the other hand conserved between even distantly related organisms



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

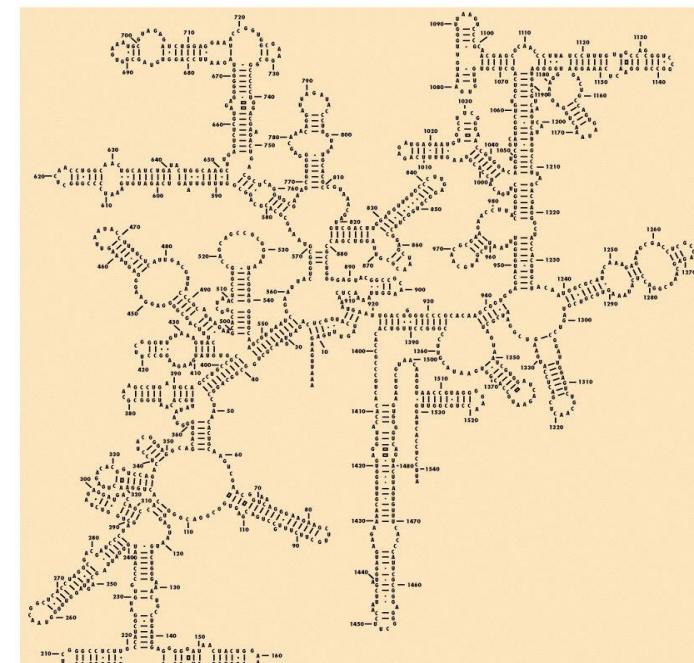


Figure 11-11c Brock Biology of Microorganisms 11/e
© 2006 Pearson Prentice Hall, Inc.

How do we differentiate **bacterial** species?

- DNA:DNA hybridisation (old fashion way)
- 16S rRNA gene sequencing
- **Whole genome sequencing**
 - **Average Nucleotide Identity (ANI)**
 - Digital DNA-DNA hybridisation values (dDDH)

ANI is a measure of nucleotide-level genomic similarity between the coding regions of two genomes

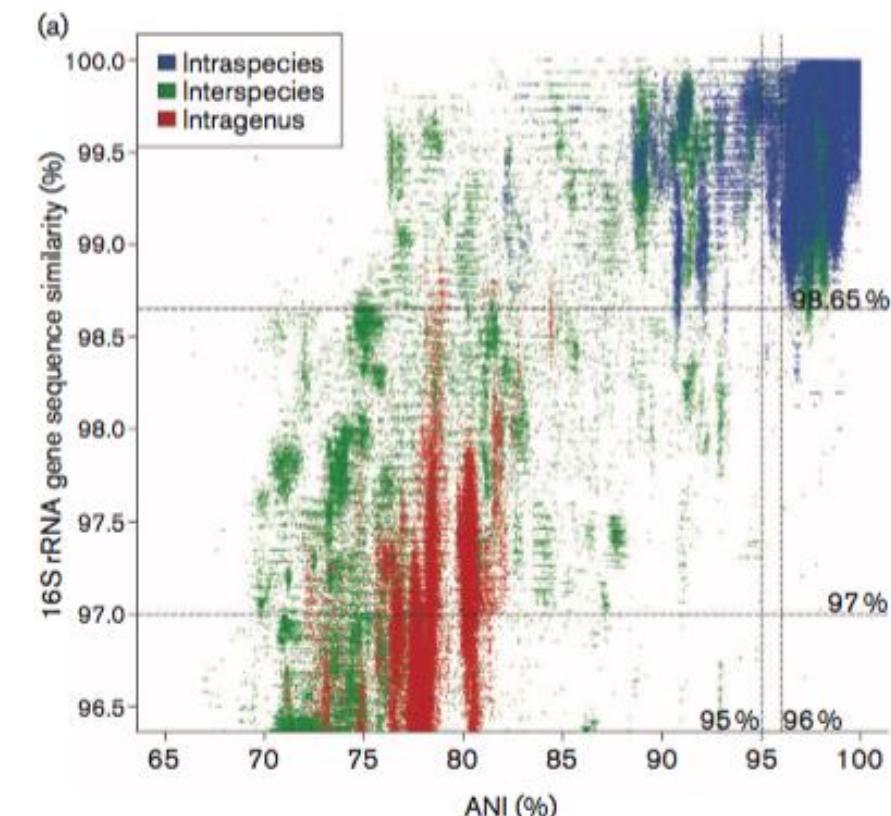
Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes

Mincheol Kim,¹ Hyun-Seok Oh,² Sang-Cheol Park² and Jongsik Chun^{1,2}

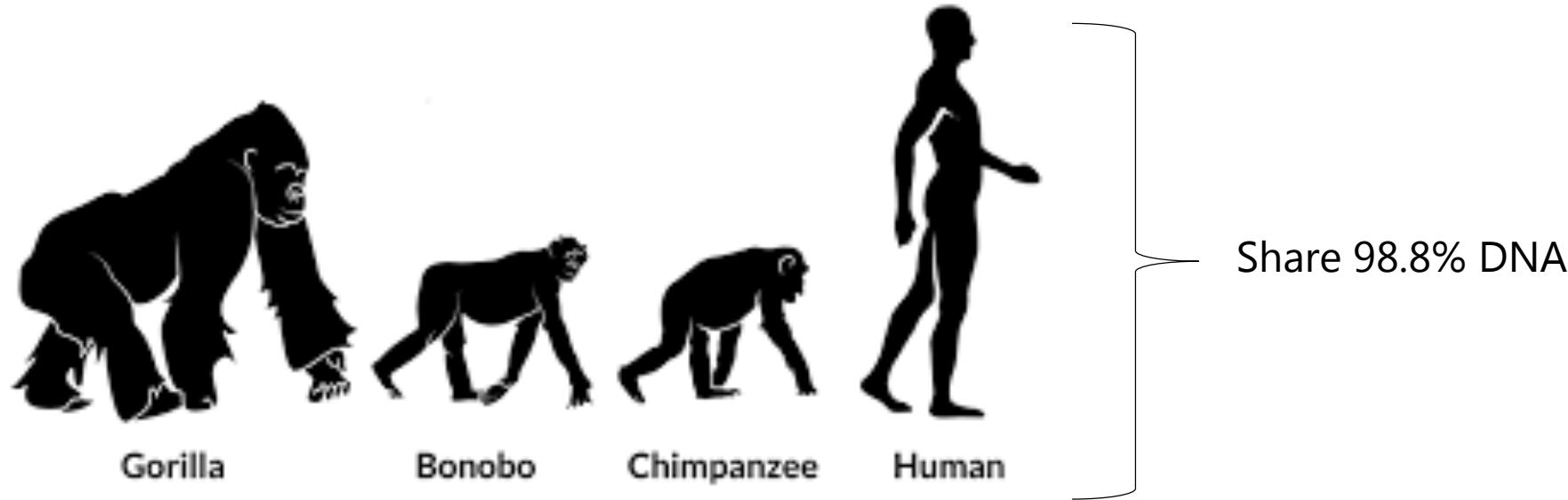
¹School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

²Interdisciplinary Program in Bioinformatics and Bioinformatics Institute, Seoul National University, Seoul 151-742, Republic of Korea

Same species ANI > 95-96%



Species concepts in bacteria vs. animals

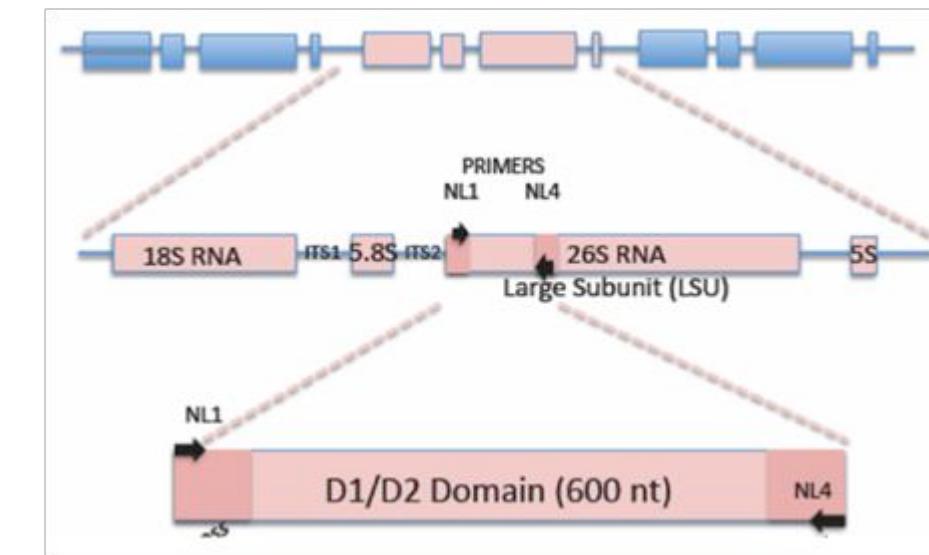
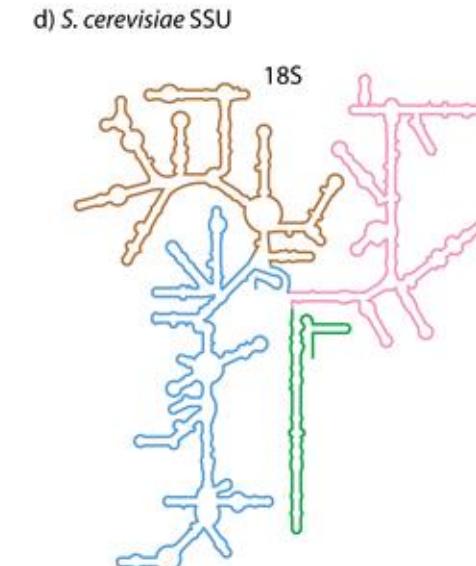
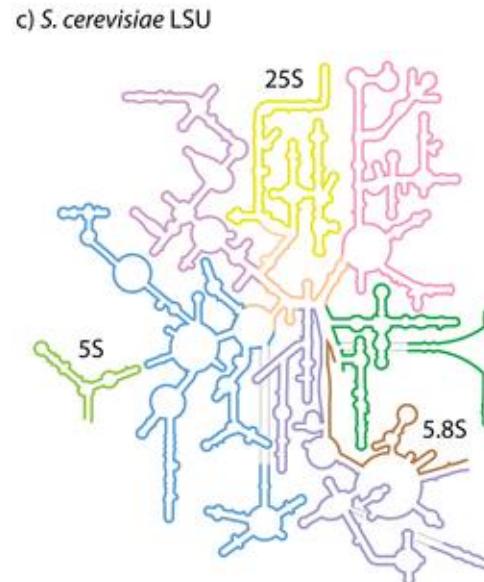


If we apply the **bacterial** species concept ($\text{ANI} > 95\text{-}96\%$) to primates, all primates would belong to the same species

How do we differentiate **yeast** and **mould** species?

- Sequence analysis of D1/D2 region (approx. 600 bp) of **26S rRNA gene**

Same species **26S rRNA ≥99%**



Petrov AS, Bernier CR, Gulen B, Waterbury CC, Hershkovits E, et al. (2014) Secondary Structures of rRNAs from All Three Domains of Life. PLOS ONE 9(2): e88222. <https://doi.org/10.1371/journal.pone.0088222>
<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0088222>

How do we differentiate **yeast** and **mould** species?

- Sequence analysis of D1/D2 region (approx. 600 bp) of **26S rRNA gene**

Same species **26S rRNA ≥99%**

- Internal Transcribed Spacer (ITS) regions**

Same species 0-3 nt. differences → 100-99% identical sequences

Same species **ITS >99%**

Different species >3-4 nt. differences → <99% identical sequences

- Average Nucleotide Identity (ANI)**

Same species **ANI >95%**

Only sometimes: ability to mate (sexual reproduction)

Genes sequenced for species identification of yeasts

Conserved genes

- **18S rRNA | 26S rRNA**
- **Internal Transcribed Spacer (ITS1 | ITS2)**
- Laccase gene (*CNLAC1*)
- Gene for actin (*ACT1*)
- Genes of subunits of RNA polymerase II (*RBP1* and *RPB2*)
- Gene of translation elongation factor-1 α (*TEF1 α*)
- Mitochondrial genes for ATP synthetase subunit 6 (*ATP6*) and large subunit rRNA

Taxonomy constantly evolving

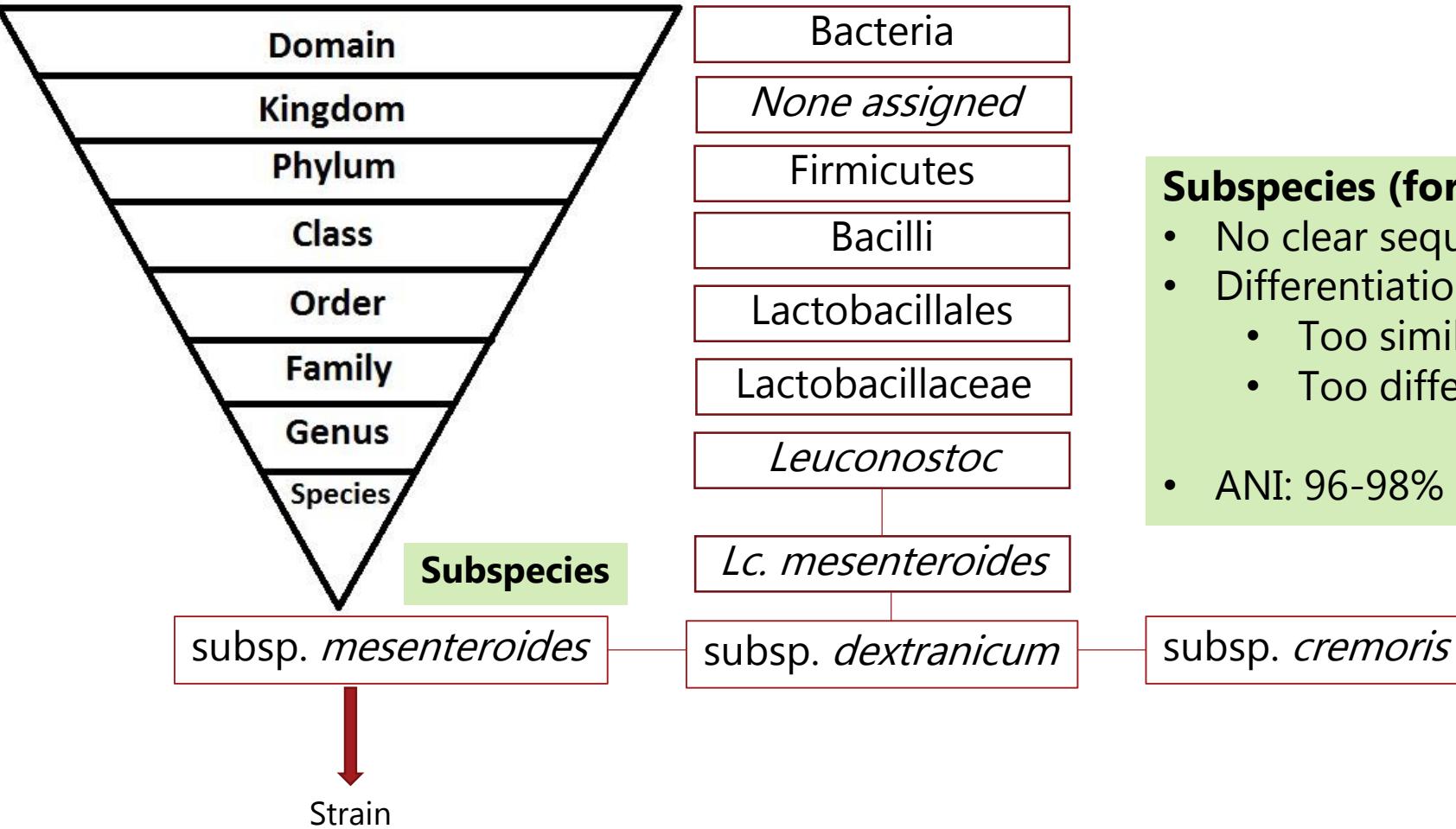
Updated list of recognized species

- Bacteria: List of Prokaryotic names with Standing in Nomenclature
<https://www.bacterio.net/>

Lactobacillus: <http://lactobacillus.uantwerpen.be/>

- Yeasts: <https://theyeast.org/>

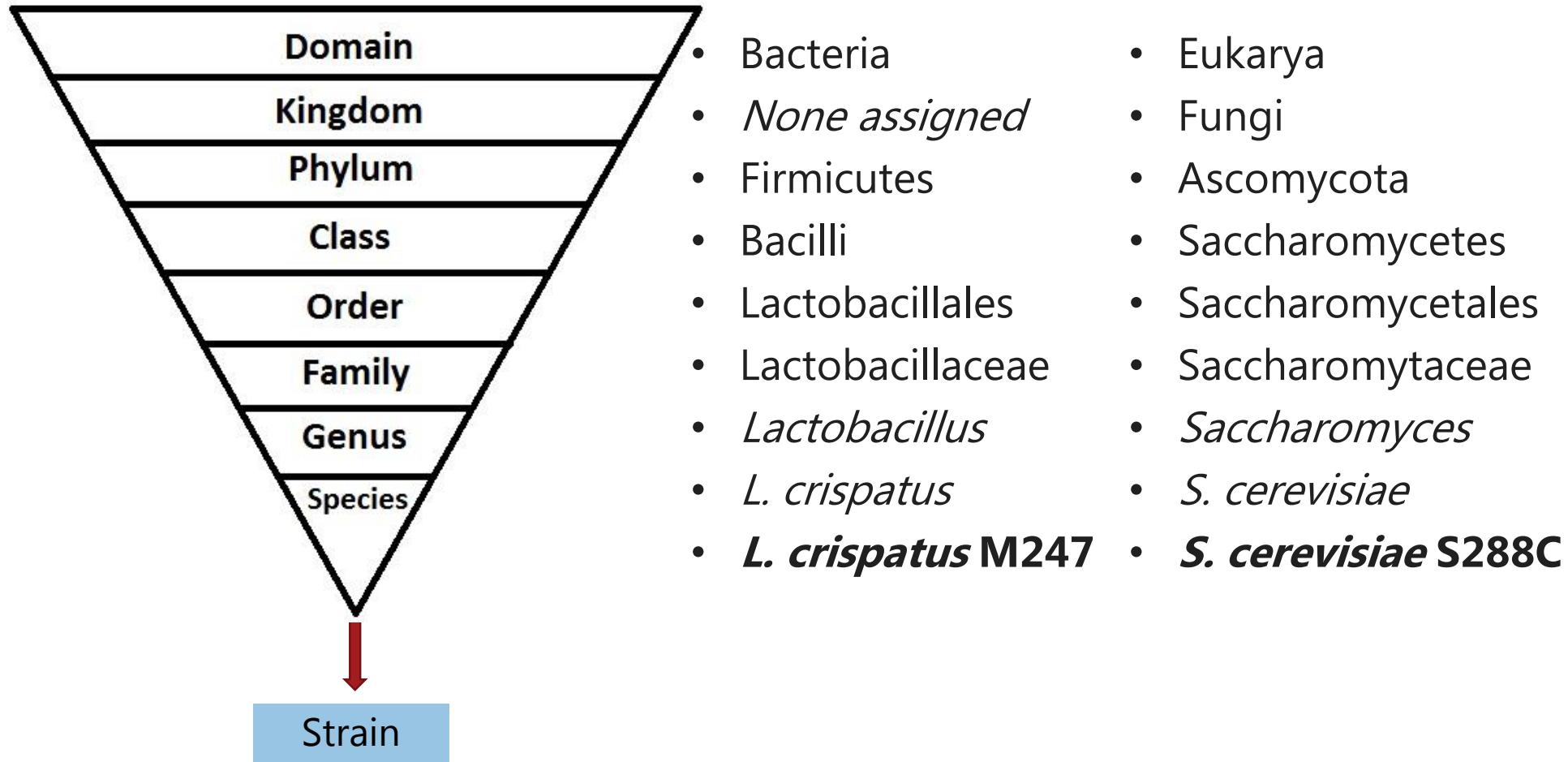
4. Subspecies, variants and strains



Subspecies (for bacteria) and variants (for yeasts)

- No clear sequence cut-off yet!
- Differentiation based on phenotype
 - Too similar to be different species
 - Too different to be same strains
- ANI: 96-98%

Microbial taxonomy: strains



Strain typing

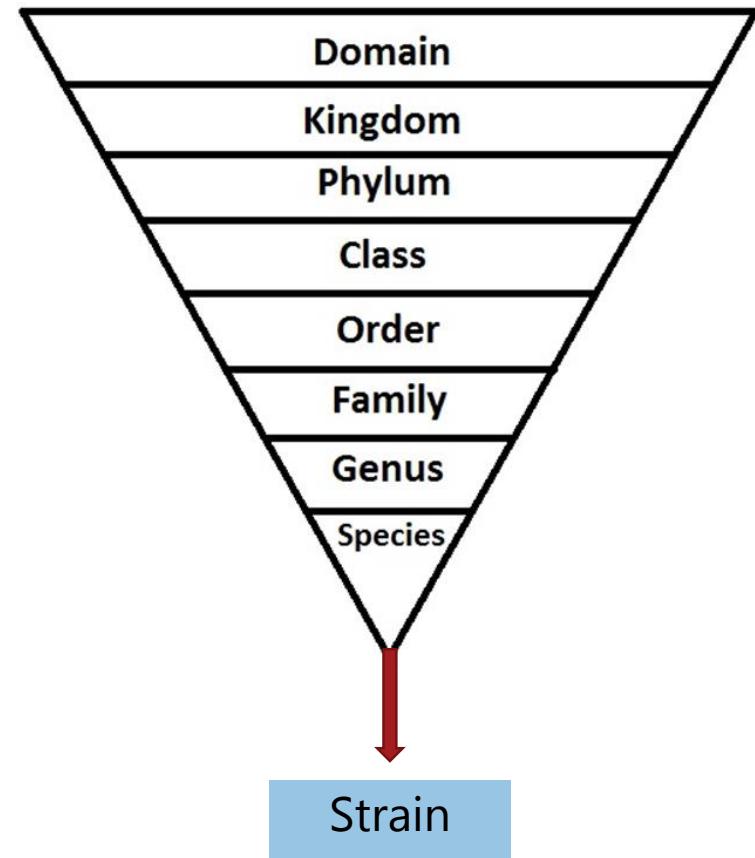
The process of strain differentiation is called **typing**. An isolate or a group of isolates that can be distinguished from other isolates of the **same** genus and **species** by

(a) **phenotypic characteristics**

- carbohydrate fermentation profile
- phage sensitivity profile
- protein expression profile

(b) **genotypic characteristics**

- Average Nucleotide Identity (ANI) – ≥99%
- Sequence in selected genes (Multi-Locus Sequence Typing)
- PFGE | REP-PCR | ON-REP-SEQ



Strain cut-off level depends on species and application (food fermentation, epidemiology)!

J. Med. Microbiol. — Vol. 49 (2000), 397–401
© 2000 The Pathological Society of Great Britain and Ireland
ISSN 0022-2615

REVIEW ARTICLE

Strain, clone and species: comments on three basic concepts of bacteriology

L. DIJKSHOORN, B. M. URsing* and J. B. URsing†

Type strains and reference strains

Type strain: the strain on which the description of a species is based

Reference strain: a strain widely used within the community, often available in a curated culture collection

<https://www.bacterio.net/>

Species *Lactococcus lactis*

◀ parent «siblings» children ▶

① Name: *Lactococcus lactis* (Lister 1873) Schleifer et al. 1986

① Category: Species

① Proposed as: comb. nov.

① Basonym: ["Bacterium lactis"](#) Lister 1873

① Etymology: *lactis*, L. gen. neut. n. *lactis*, of milk

① Gender: masculine

① Type strain: ATCC 19435; CCUG 32211; CCUG 7980; CIP 70.56; DSM 20481; HAMBI 1591; JCM 5805; LMG 6890; NBIMCC 4000; NBRC 100933; NCDO 604; NCFB 604; NCIB 6681; NCIMB 6681; NCTC 6681; VKM B-1662

① See detailed strain information at [BacDive](#)

① Conduct genome-based taxonomy at [TYGS](#)

① 16S rRNA gene: M58837 Analyse FASTA ENA NCBI

① Effective publication: Schleifer KH, Kraus J, Dvorak C, Kilpper-Balz R, Collins MD, Fischer W. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. *Syst. Appl. Microbiol.* 1985; 6:183-195.

① IJSEM list: Anonymous. Validation list no. 20. Validation of publication of new names and new combinations previously effectively published outside the IJSB. *Int J Syst Bacteriol* 1986; 36:354-356.

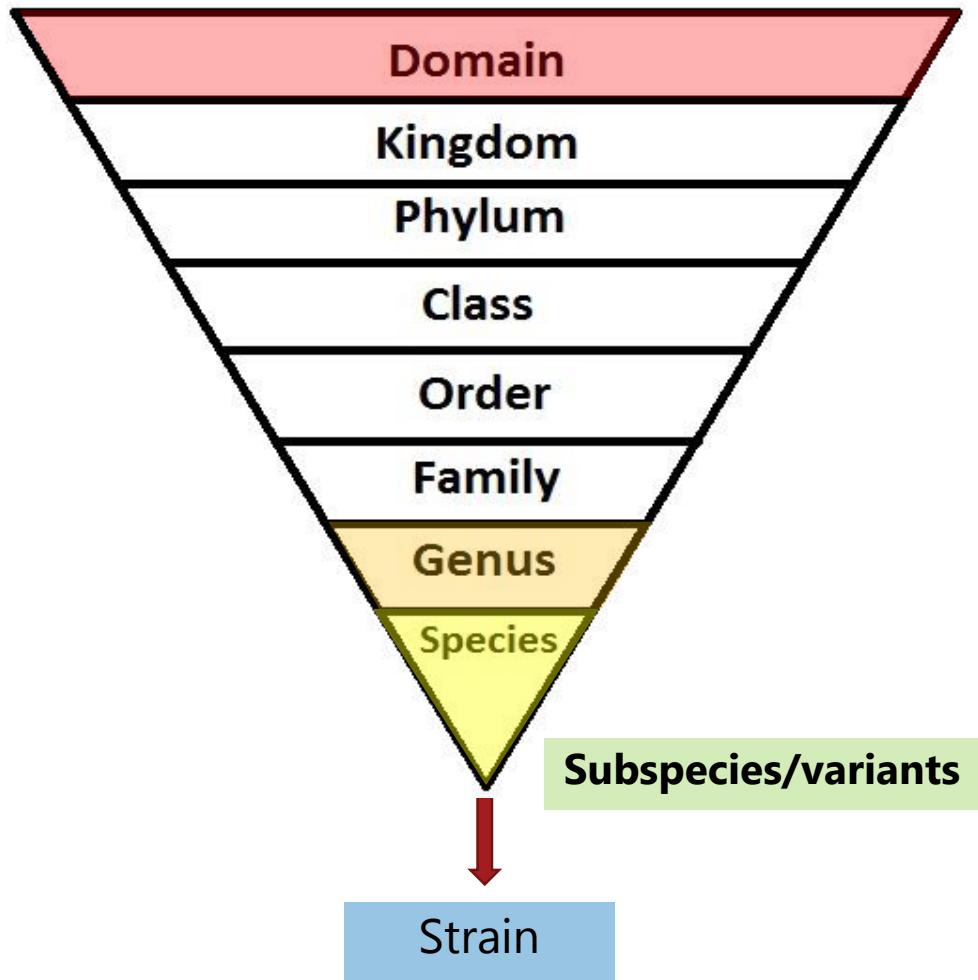
① Nomenclatural status: validly published under the ICNP

① Taxonomic status: correct name

① ▼ Synonyms:

Name ▾	Kind
"Bacterium lactis" Lister 1873	homotypic synonym, not validly published, basonym of name in Approved Lists
Streptococcus lactis subsp. diacetilactis (ex Matuszewski et al. 1936) Garvie and Farrow 1982	heterotypic synonym, validly published under the ICNP, distinct rank
Streptococcus lactis (Lister 1873) Löhnis 1909 (Approved Lists 1980)	homotypic synonym, validly published under the ICNP
Lactobacillus xylosus Kitahara 1938 (Approved Lists 1980)	heterotypic synonym, validly published under the ICNP

What did you learn today?



- Taxonomy and phylogeny
- Three domains of life
 - Archaea vs. Bacteria vs. Eukarya
 - Gram-positive vs. Gram-negative
- The species concept in microbiology
 - 16S rRNA gene
 - The 26S rRNA gene, D1/D2 region
 - Average Nucleotide Identity (ANI)
- Subspecies, variants and strains

Main concepts

- | | | |
|----------------------|-----------------------------------|--------------------|
| • Taxonomy | • Average Nucleotide Identity | • Strain |
| • Phylogeny | • (digital) DNA-DNA hybridisation | • Typing |
| • Nomenclature | • Subspecies | • Type strain |
| • Species | • Variant | • Reference strain |
| • 16S rRNA, 26S rRNA | | |

EXTRA

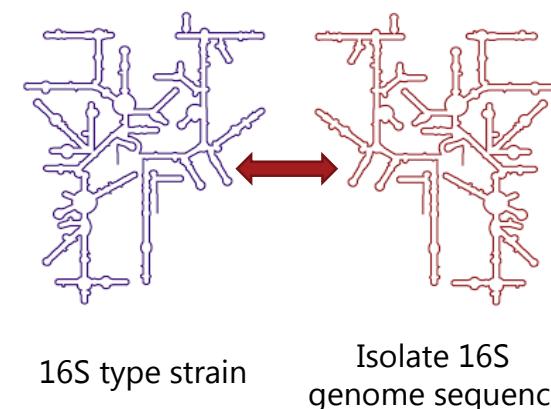
Minimal standards for the use of genome data for the taxonomy of prokaryotes

Step 1: Sequencing details



1. The **sequencing** instrument, library reagents and method for genome assembly should be described in detail.

Step 2: Check contamination (16S)

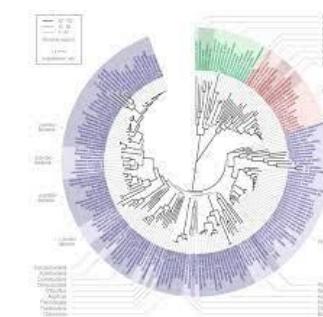


16S type strain Isolate 16S genome sequence

2. Potential **contamination** should be checked by comparing 16S sequences

Step 3: ANI/dDDH, phylogeny

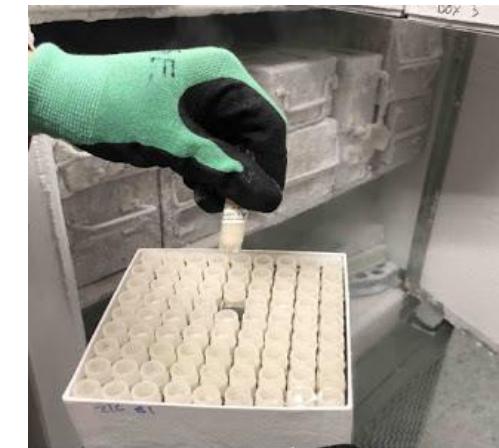
Species: ANI / dDDH
Genus: ANI / dDDH + phylogeny



3. For the **proposal of new species**, ANI/dDDH should be calculated with all related species

For the classification of **genera**, you should also use **phylogeny**

Step 4: Database deposit



4. The final genome assembly should be **deposited in a publicly accessible database**.

Tools for calculation of ANI/dDDH/phylogeny

Table 1. Web-services and standalone software tools for taxonomic purposes

Algorithm	Function	Type	URL/Reference
OrthoANI with usearch	Calculation of ANI	Standalone	https://www.ezbiocloud.net/tools/orthoaniu [9]
OrthoANI with usearch	Calculation of ANI	Web service	https://www.ezbiocloud.net/tools/ani [9]
Genome-to-Genome Distance Calculator	Calculation of dDDH	Web service	http://ggdc.dsmz.de/ggdc.php/ [7]
ANI calculator	Calculation of ANI	Web service	http://enve-omics.ce.gatech.edu/ani/
JSpecies	Calculation of ANI	Standalone	http://imedea.uib-csic.es/jspecies/ [5]
JSpeciesWS	Calculation of ANI	Web service	http://jspecies.ribohost.com/ [30]
CheckM	Checking contamination	Standalone	http://ecogenomics.github.io/CheckM/ [29]
ContEst16S	Checking contamination	Web service	https://www.ezbiocloud.net/tools/contest16s [28]
BBMap	Calculation of sequencing depth of coverage	Standalone	https://sourceforge.net/projects/bbmap/
Amphora2	Phylogenomic treeing	Standalone	http://wolbachia.biology.virginia.edu/WuLab/Software.html [21]
BIGSdb	Phylogenomic treeing	Standalone	https://pubmlst.org/software/database/bigsdb/ [31]
bcgTree	Phylogenomic treeing	Standalone	https://github.com/iimog/bcgTree [32]
Phylophlan	Phylogenomic treeing	Standalone	https://huttenhower.sph.harvard.edu/phylophlan [22]
UBCG	Phylogenomic treeing	Standalone	https://www.ezbiocloud.net/tools/ubcg

(no need to know the tools)

Methodologies for identification and typing

A microbiologist's toolbox

Lene Jespersen

KØBENHAVNS UNIVERSITET

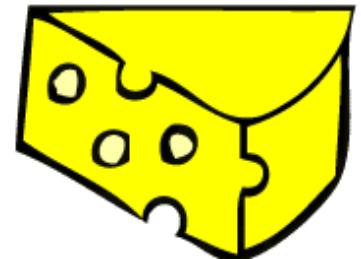


Intended leaning outcomes

- Get to know how different microbial identification and typing methods works
- Apply this knowledge to comprehend microbial identification and taxonomy
- Reflect on the application possibilities of the different methods for microbial identification and typing

Background

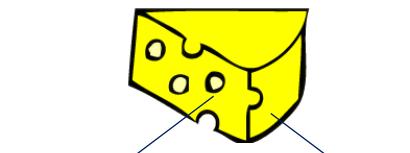
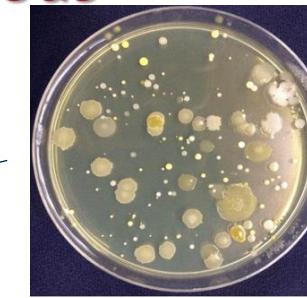
- When you want to know what microorganisms are in a fermented food
→ **IDENTIFICATION**
 - Often identification is coupled with quantification (predominant/minor microorganisms)
- Many different methods are available
 - Make an overview of the most used methods
 - Elaborate the steps of the methods → insights into the structure/mechanistic
- All methods have strengths and limitations



Note: typing is identification of isolates below species level

Methodology overview

Culture dependent methods



Plating

Isolation
↓
Purification → Pure culture



Classical

Macro/micro morphology

Phenotypic tests:
fermentation,
assimilation,
growth conditions

Finger-print based:
rep-PCR,
RAPD,
RFLP,
PFGE

Based on PCR

Sequence based:
rRNA gene, MLST, MLSA

Culture independent methods

Direct extraction of DNA or RNA

Molecular methods

PCR or qRT-PCR

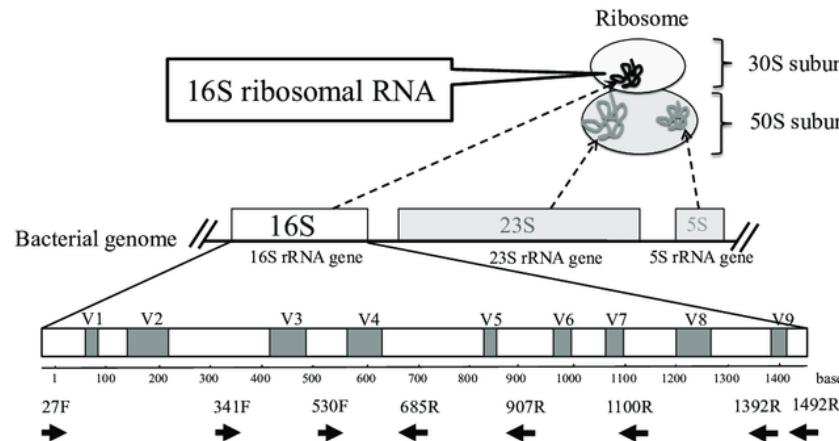
Species specific PCR
Next generation sequencing

Biochemical identification
MALDI-TOF
FTIR

Sequencing of rRNA gene (16S, 26S or ITS)

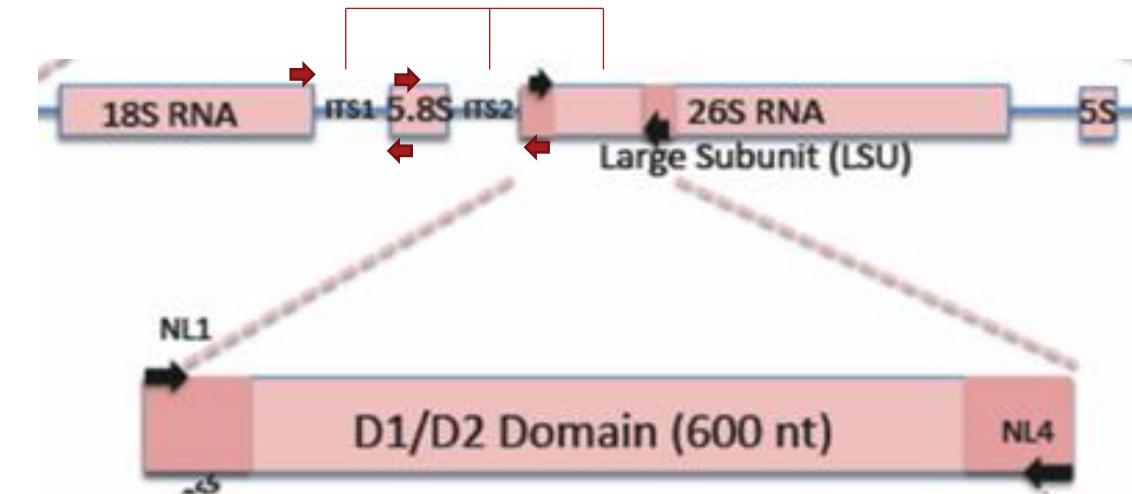
Sequencing method for identification of microorganisms

16S rRNA gene –Bacteria



Yeast (26S rRNA gene or ITS)

Variable



- Extremely powerful method
- Universal primers different for bacteria and yeasts
- Still widely used in identifications, e.g. coupled with rep-PCR, RAPD, and classical methodologies
- Resolution, nearly always to species level

Yeasts:

Positions 1-126 + 265-422:

Evolutionary conserved regions

Positions 127-264 (D1) + 423-636 (D2):

Variable regions

More than 1.0 % deviation in the nucleotides of the D1/D2 region indicates a new species

Alignment

Score = 2448.7 bits (1235), Expect = 0E00
Identities = 1235/1235 (100), Gaps = 0/1235 (0)
Strand = Plus/Minus

Query	1	GTGTACAAGGCCCGGGAACGTATTACCGCGGCGTGCCTGATCCGCGATTACTAGCGATT	60
Sbjct	1345	GTGTACAAGGCCCGGGAACGTATTACCGCGGCGTGCCTGATCCGCGATTACTAGCGATT	1286
Query	61	CGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACGTGAGAATGGTTTAAGAGATTA	120
Sbjct	1285	CGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACGTGAGAATGGTTTAAGAGATTA	1226
Query	121	GCTAAACATCACTGTCTCGGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAG	180
Sbjct	1225	GCTAAACATCACTGTCTCGGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAG	1166
Query	181	GTCATAAGGGCATGATGATTGACGTACATCCCCACCTTCCCGTTATCACCGGCAG	240
Sbjct	1165	GTCATAAGGGCATGATGATTGACGTACATCCCCACCTTCCCGTTATCACCGGCAG	1106
Query	241	TCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAAATAGGGTTGCGCTCGTTGCGG	300
Sbjct	1105	TCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAAATAGGGTTGCGCTCGTTGCGG	1046
Query	301	GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCACATGCACCACTGTATCCGT	360
Sbjct	1045	GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCACATGCACCACTGTATCCGT	986
Query	361	GTCCCGAAGGAACCTCCTATCTCTAGGAATAGCACGAGTATGTCAAGACCTGGTAAGGTT	420
Sbjct	985	GTCCCGAAGGAACCTCCTATCTCTAGGAATAGCACGAGTATGTCAAGACCTGGTAAGGTT	926
Query	421	CTTCGCGTTCTCGAATTAAACCACATGCTCCACCGCTGTGCGGGCCCCCGTCAATT	480
Sbjct	925	CTTCGCGTTCTCGAATTAAACCACATGCTCCACCGCTGTGCGGGCCCCCGTCAATT	866

Identification of yeasts based on homology search

Table 4 Identification of yeast species in fermented liquid feed by sequencing of the D1/D2 region of the 26S rRNA gene

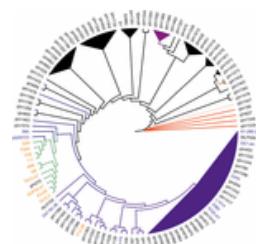
Groups	Number of isolates sequenced	Length D1/D2 sequence	Identities	Similarity to GenBank sequence (%)	GenBank accession no.	Closest related yeast species
I	49	601	570/570	100.0	GQ222344	<i>C. milleri/C. humilis</i>
II	13	599	597/597	100.0	GQ222345	<i>Kazachstania exigua</i> ^a
III	11	472	467/473	98.7	GQ222346	<i>Candida pararugosa</i>
IV	4	594	570/571	99.8	GQ222347	<i>Kazachstania bulderi</i> ^b
V	2	603	572/572	100.0	GQ222348	<i>Kazachstania unispora</i> ^c
VI	2	662	520/524	99.2	GQ222349	<i>Pichia burtonii</i>
VII	1	602	601/601	100.0	GQ222350	<i>Saccharomyces cerevisiae</i>
VIII	2	587	573/573	100.0	GQ222361	<i>Kregervanrija fluxuum</i>
IX	4	625	607/624	97.1	GQ222351	<i>Trichosporon dulcitum</i>
X	2	495	467/470	99.4	GQ222353	<i>Pichia deserticola</i>
XI	2	573	554/554	100.0	GQ222354	<i>Candida silvae</i>
XII	1	432	404/404	100.0	GQ222355	<i>Geotrichum fragrans</i>
XIII	1	548	546/549	99.5	GQ222356	<i>Pichia membranifaciens</i>
XIV	1	546	546/546	100.0	GQ222357	<i>Trichosporon brassicae</i>
XV	1	563	561/563	99.6	GQ222358	<i>Debaryomyces hansenii</i>
XVI	1	535	535/535	100.0	GQ222359	<i>Yarrowia lipolytica</i>
XVII	1	534	519/536	97.2	GQ222360	<i>Geotrichum klebahnii</i>
XVIII	1	600	597/599	99.6	GQ222362	<i>Pichia fermentans</i>

^a Formerly named as *Saccharomyces exiguous*

^b Formerly named as *Saccharomyces bulderi*

^c Formerly named as *Saccharomyces unisporus*

Remember always to cross check with the phenotypic criteria !!!
 The database is not better than the person submitting the sequence ;-)



“Classical” methods: macro- and micro morphology

- Macro-morphology (describing appearance of colonies)
- Micromorphology (describing cell shape and for yeast vegetative formation and spore morphology)
- Microscopy (light/phase contrast)
 - Rapid
 - Simple
 - Detection limit
 - Live/dead?



Mainly for species identification

"Classical" methods - phenotypic tests

- Phenotypic tests on plates or in liquid broth
 - Simple
 - Low detection limit (in principle)
 - Mixed cultures
 - Selective substrates
 - low pH
 - antibiotics
 - high ethanol
 - organic acids
 - high sugar
 - Selective growth conditions
 - temperature (high/low)
 - Assimilation of carbohydrates/nitrate
 - Fermentation of carbohydrates



Mainly for species identification

An example of phenotypic/technological characterisation of brewing yeast strains

- Flocculence
(high, moderate, weak, non-flocculent)
- Reproduction
(adequate, inadequate)
- Attenuation
(high, moderate, weak)
- Aromatic properties
(aromatic, slightly aromatic, non-aromatic)
- Off flavours
(clean, unclean; sulphury, diacetyl)
- For starter cultures some phenotypic traits will be important technical characteristics and are therefore of significance relevance in the identification at strain level

Example:

Highly flocculent:

$$Y_{10} < 1.5$$

Moderate flocculent:

$$Y_{10} = 1.5 \text{ to } 2.5$$

Weakly flocculent:

$$Y_{10} > 2.5$$

Y_{10} = g yeast per litre in suspension on day 10

Digit code describing the specific characteristics can be made

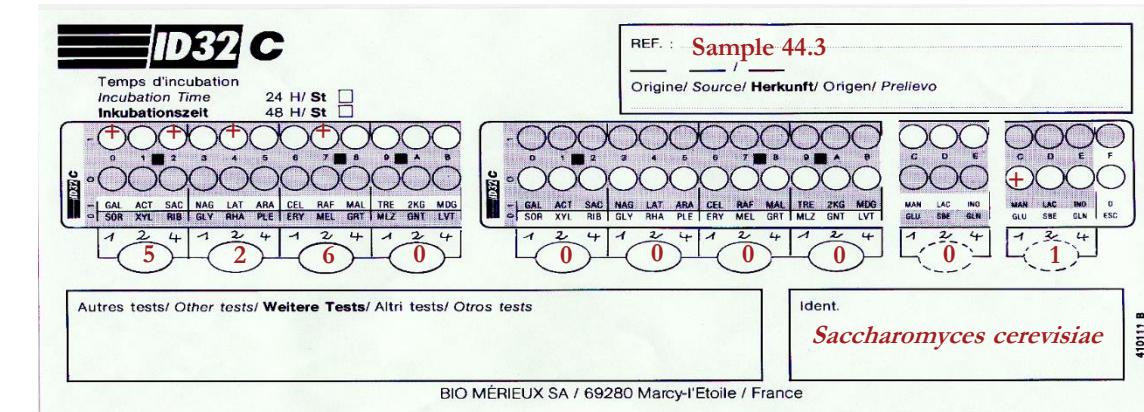


Identification systems - API

- Simple identification techniques
- Used in small companies for simple identification
- Assimilation of carbon compounds

API 20C (Analytab Products, Plainview, NY)

API ID 32C (-do-)



- Enzymic activity
- MicroScan (Baxter Diagnostic Inc., West Sacramento, CA)
- API YeastIdent. (Analytab Products))

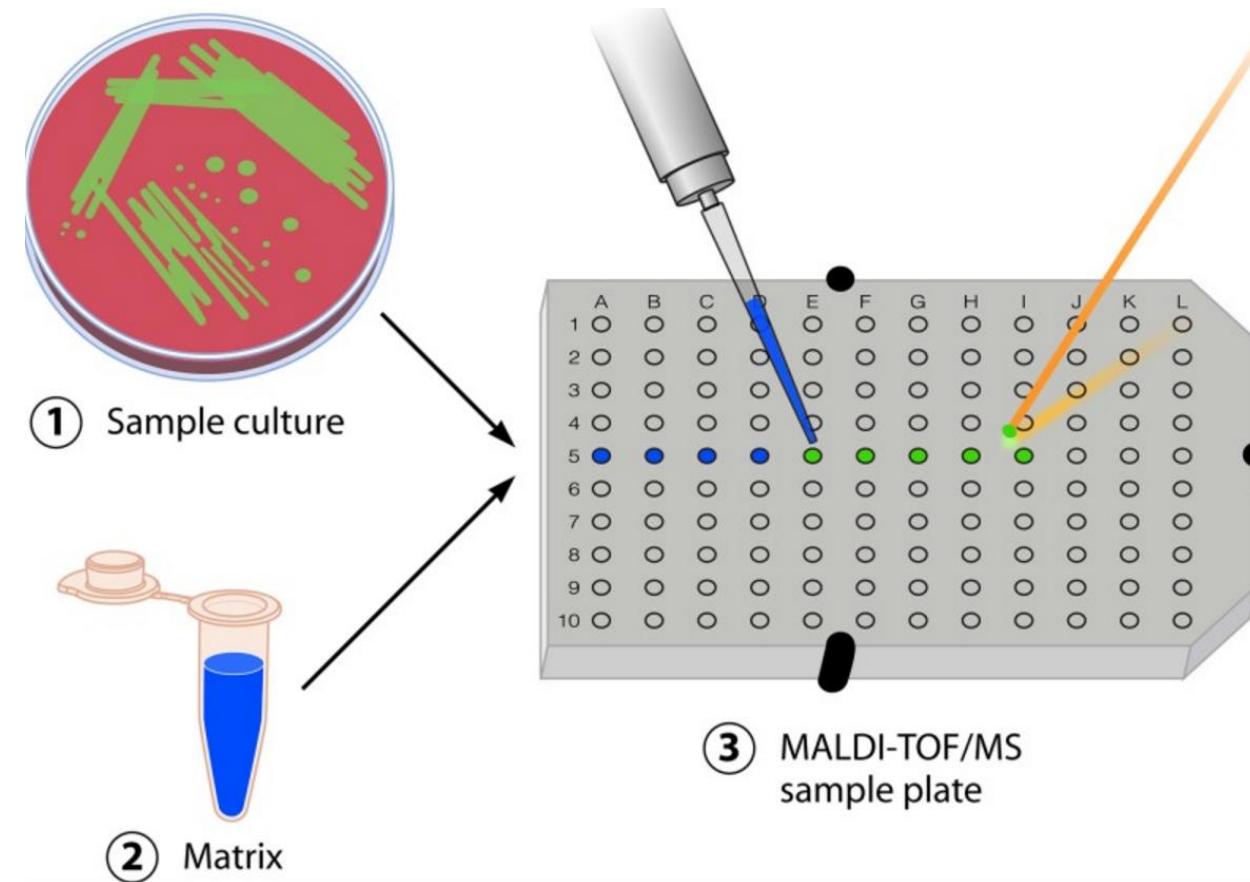


Mainly for species identification

MALDI-TOF MS (1/3)

MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry

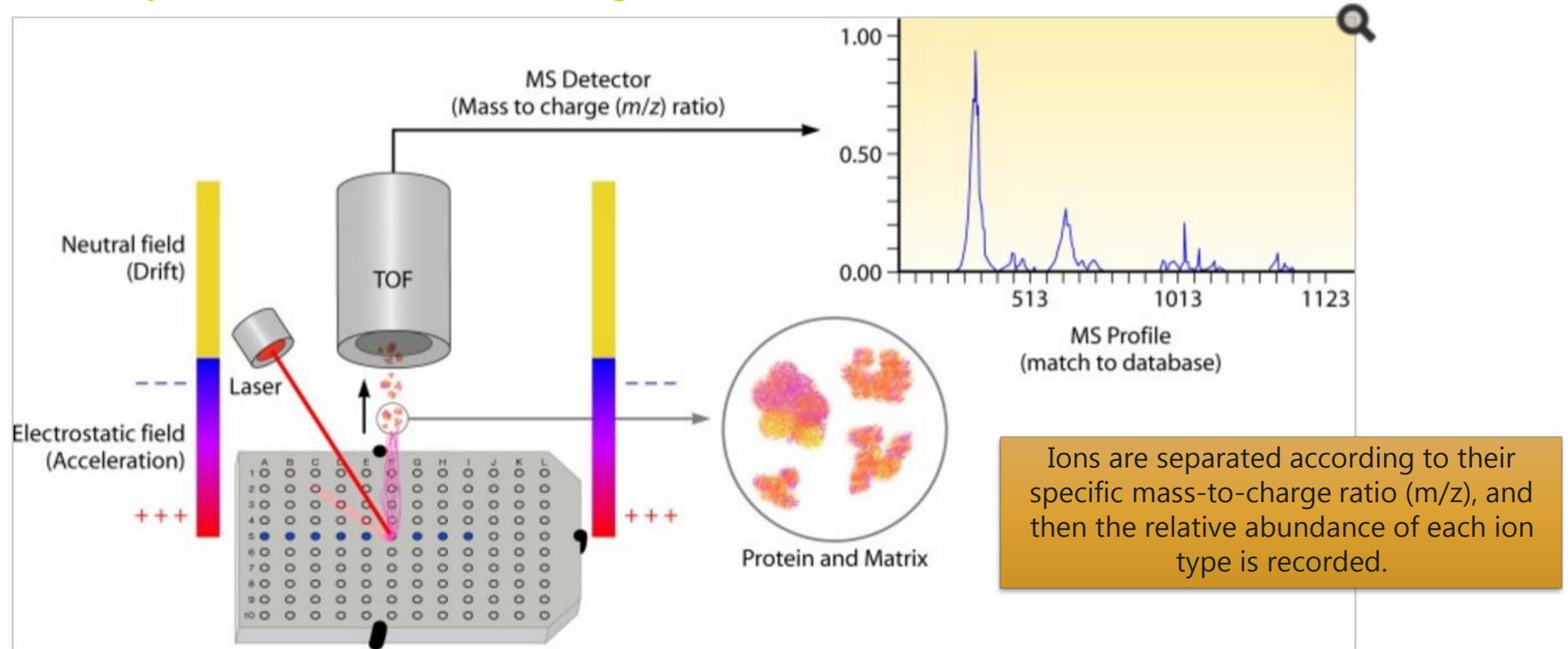
Sample preparation:



Mainly for species identification

MALDI-TOF MS (2/3)

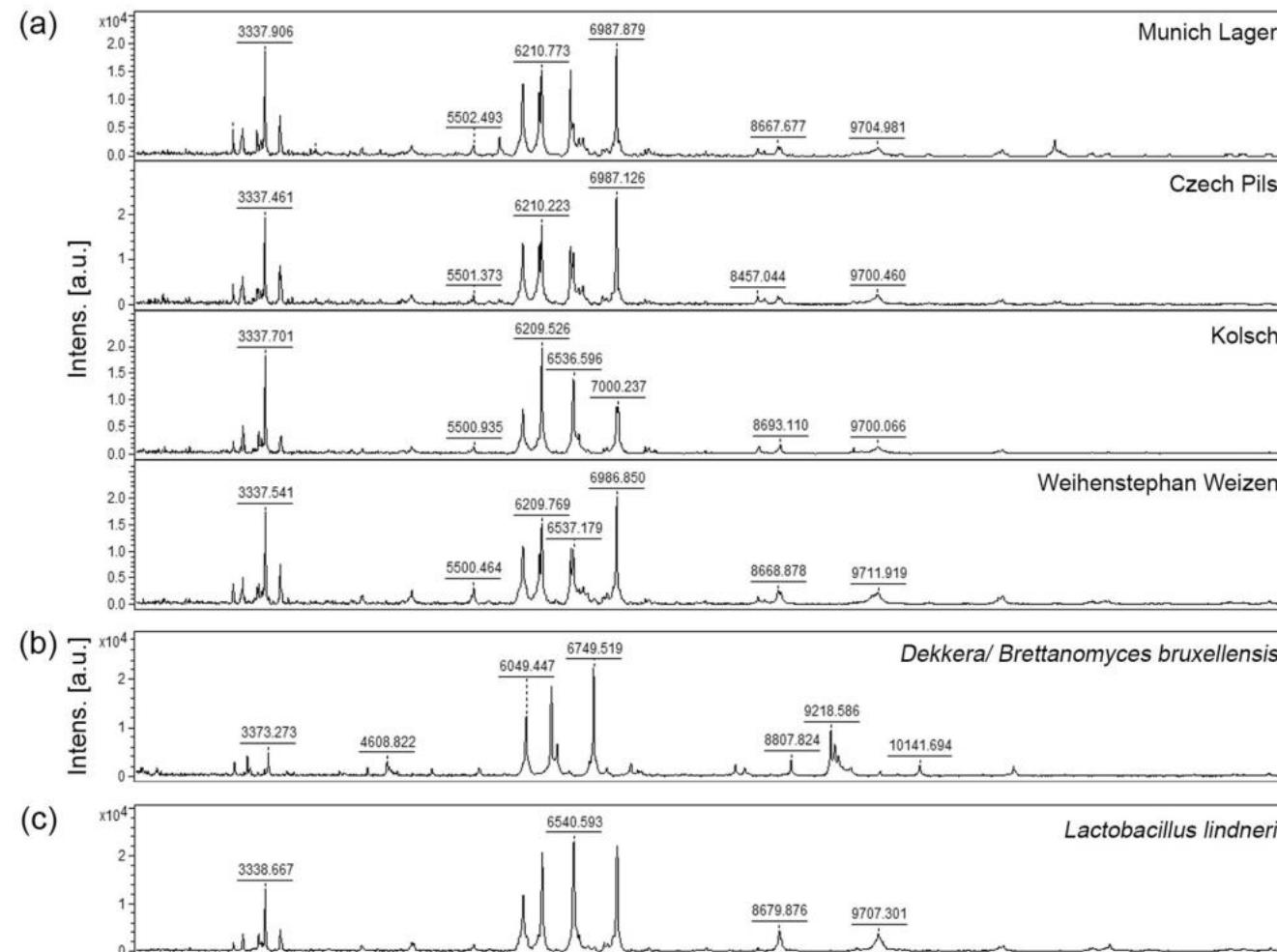
MS analysis of ionized microbiological isolates:



MALDI-TOF MS measures **complex mixtures of proteins** disclosing a unique fingerprint for each species. Since the proteins detected are basically ribosomal ones, which are constitutively expressed at very high levels, this phenotypic technique is less influenced by expression variability

Identification of yeasts by MALDI-TOF MS (3/3)

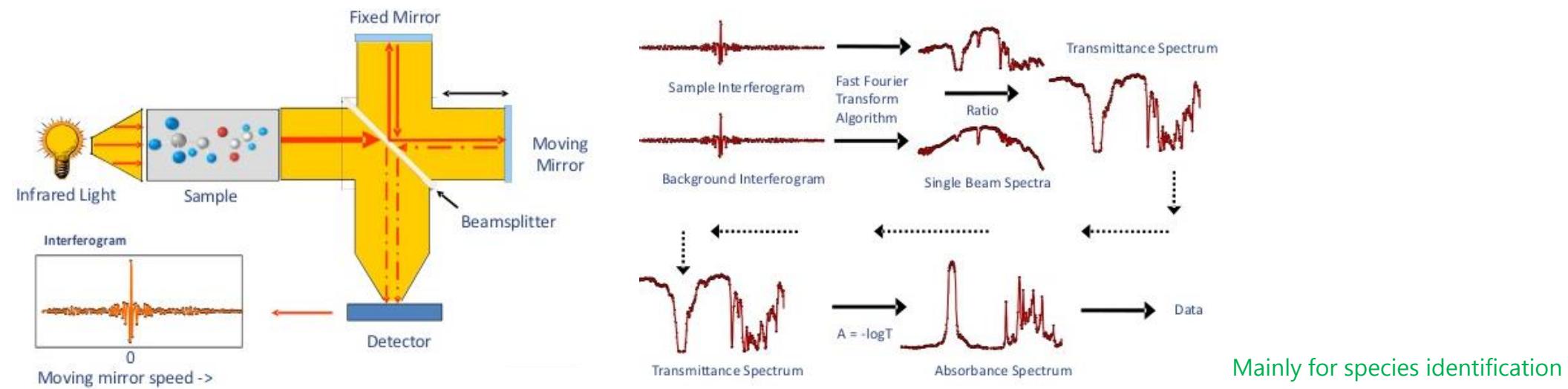
Identification of brewing contaminants:



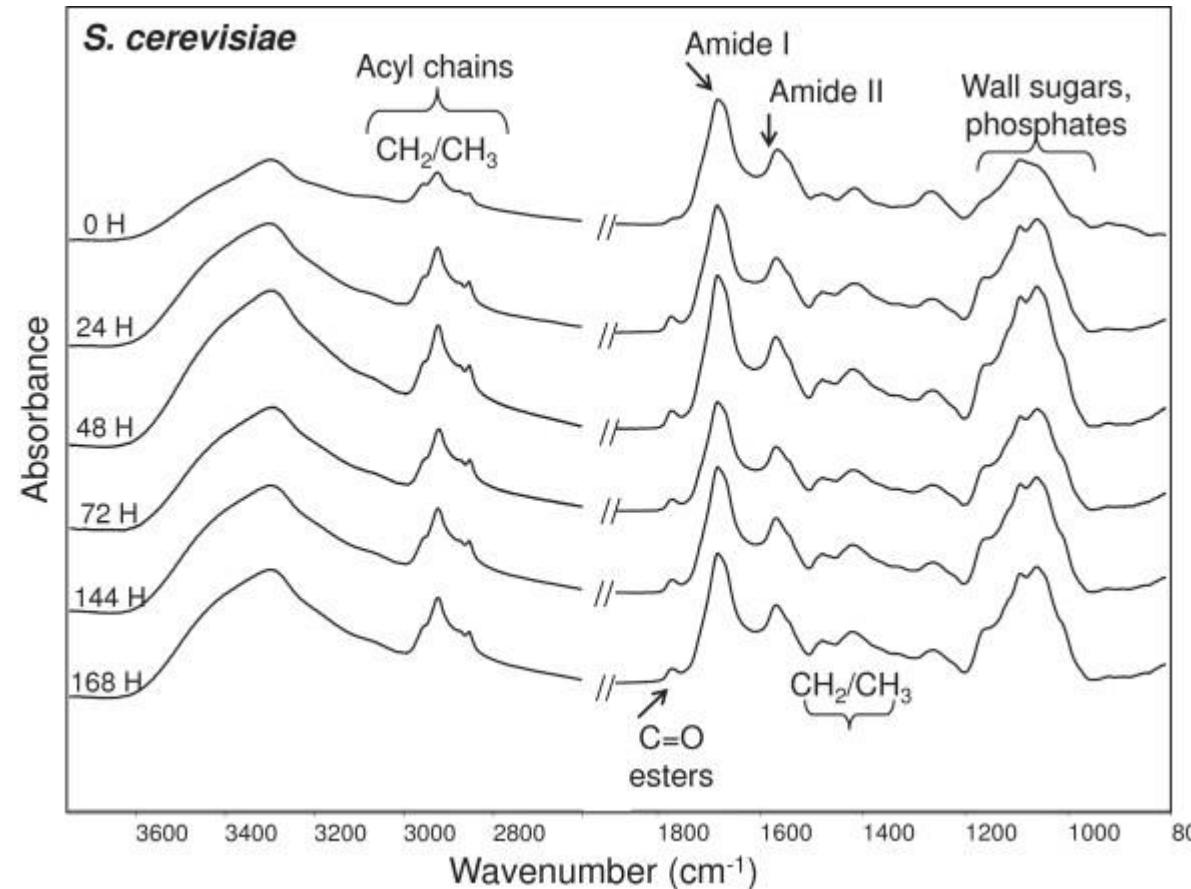
- a) commercially available brewing yeasts (1-2: lager yeasts; 3-4: ale yeasts)
b) wild yeast c) beer spoilage bacteria

Fourier Transform Infrared Spectroscopy (FTIR) (1/2)

- FTIR is based on infrared (IR) spectroscopy
- IR radiation is passed through a sample. Part of the radiation is absorbed by the sample and some is transmitted
- A molecular fingerprint (spectrum) of the sample (microbial biomass) is created based on the molecular absorption and transmission
- When compared to a database the spectrum can be used for identification of well-known microorganisms



Fourier Transform Infrared Spectroscopy (FTIR) (2/2)

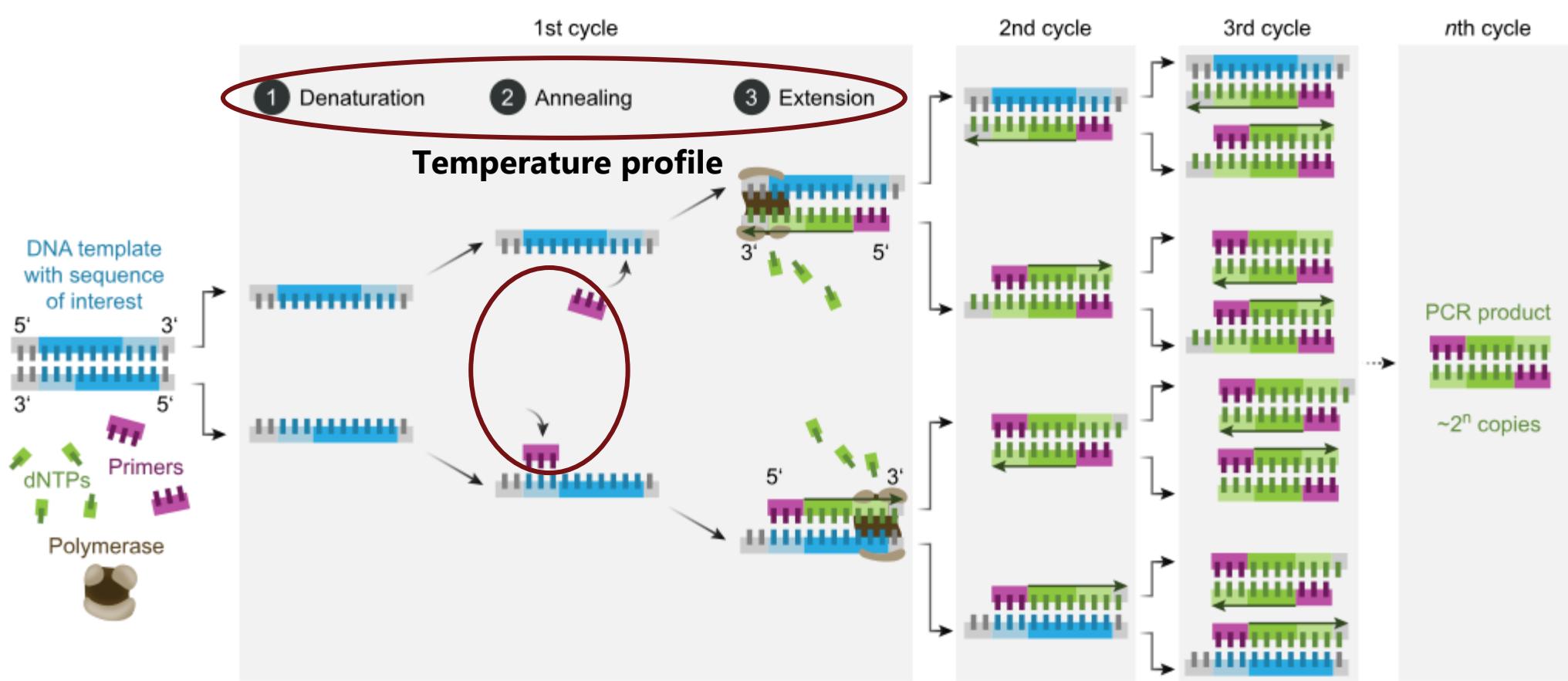




Let's take a break

PCR: Polymerase chain reaction

- Many identification (and typing) methods are based on PCR

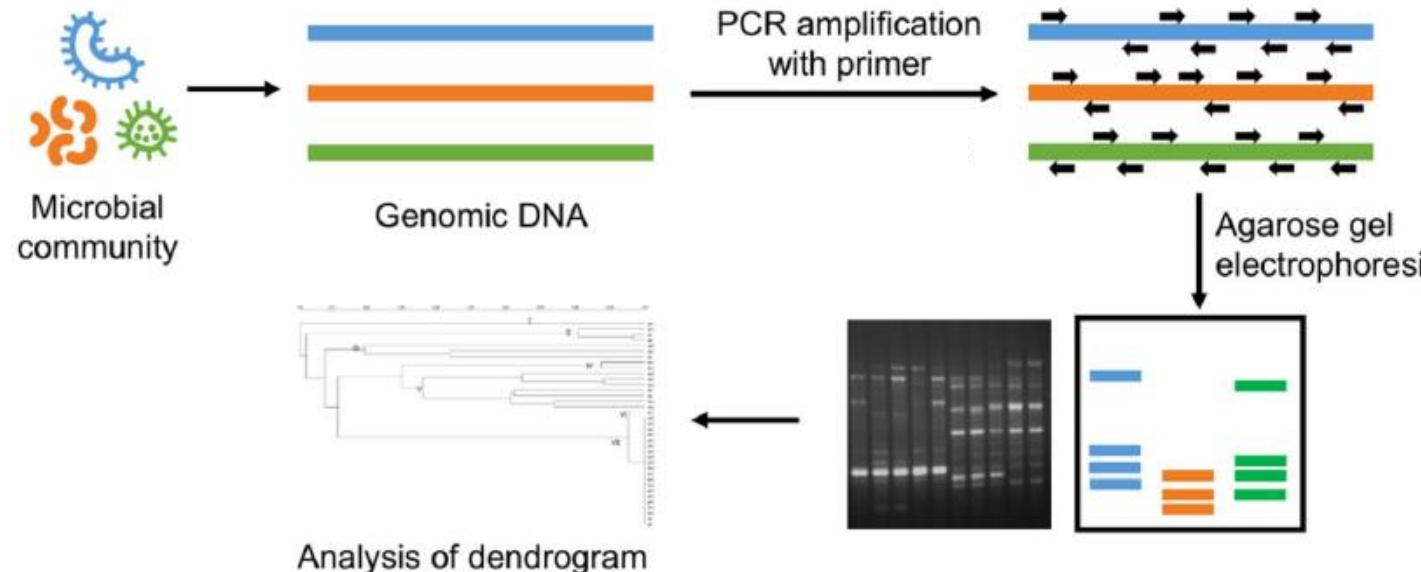


Used with many different primers for species identification and typing of strains

rep-PCR

-repetitive extragenic palindromic PCR

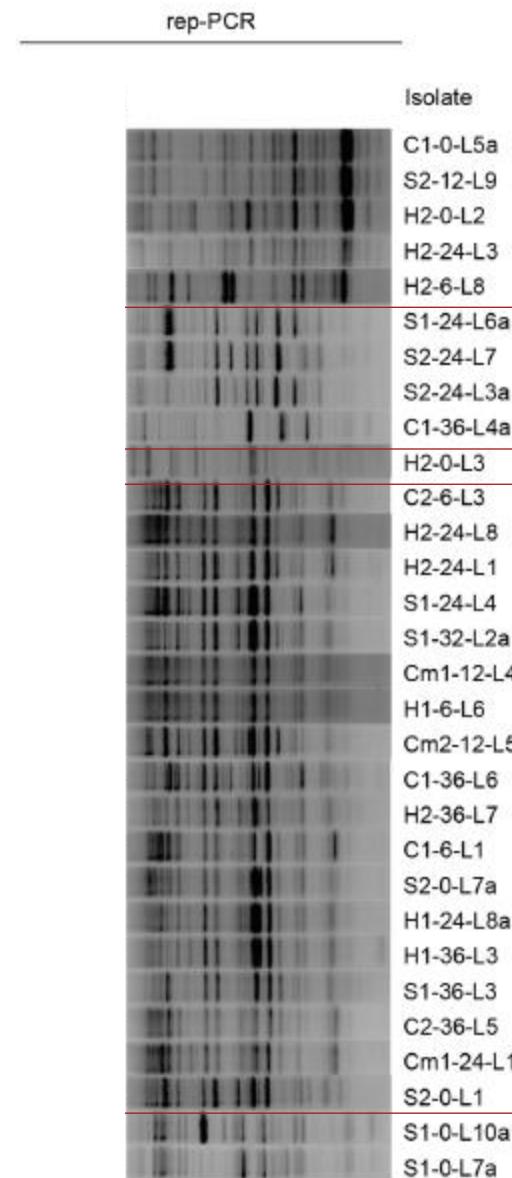
- Primers target interspersed repetitive specific DNA elements
- rep-PCR used in the lab exercise with $(GTG)_5$
- No prior knowledge of the DNA sequence is needed
- Used for initial grouping of microorganisms → reducing sequencing cost



REP primers	Size (bases)
Rep1	IIIICGICGICATCIGGC
	IIICGNCGNCATCNGGCA
ERIC-primers	ATGTAAGCTCCTGGGGATTAC
	AAGTAAGTGACTGGGGTGAACG
BOXA1R	CTACGGCAAGGCGACGCTGACG
BOXA2R	ACGTGGTTGAAGAGAGATTCG
(GTG)₅	GTGGTGGTGGTGGT
(CAC) ₄	CACACACACACACACA

rep-PCR result

- Generate a fingerprint for each isolate
- Same fingerprint = same species
- Grouping of microorganisms
- Discriminatory power depends on primer, DNA extraction method and choice of polymerase
- Should be coupled with sequencing to obtain identification
- Drawbacks:
 - Labor intensive to assign bands for grouping



RAPD - Random Amplification of Polymorphic DNA

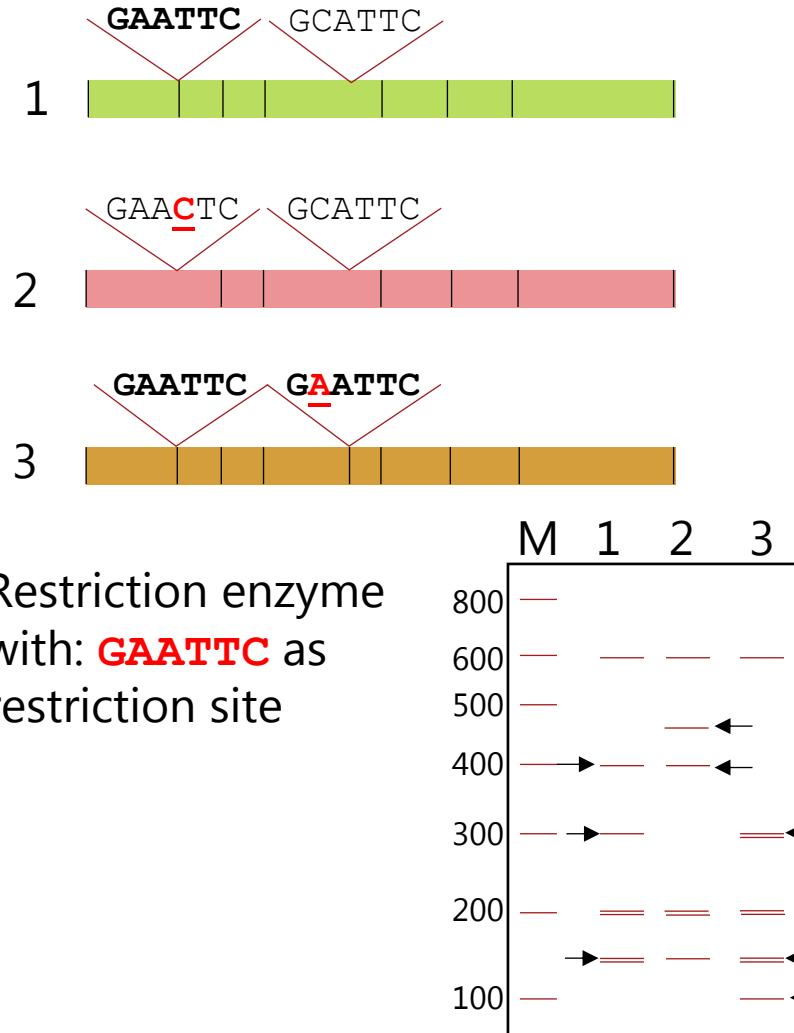
- Short primers (8–12 nucleotides), may vary from species to species
- Primers randomly hybridizes to chromosomal DNA
- The segments of DNA that are amplified are random
- RAPD is an inexpensive method for typing isolates to species and in some cases strain level
- Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains
- No knowledge of the DNA sequence for the targeted gene is required
- Major drawback: poor reproducibility

Mainly for strain typing

RAPD primers (examples)		
0910-08	CCGGCGGCG	9
940-12	ACGCGCCCT	9
955-03	CCGAGTCCA	9

RFLP

-Restriction Fragment Length polymorphism



- Restriction enzymes cleaves DNA at specific restriction sites, i.e. specific short DNA sequences
- A single mutation may remove or generate a restriction site
→single nucleotide polymorphism (SNP)
- PCR of e.g. 16S/26S rRNA gene or another gene followed by digestion with restriction enzyme
- Gel to visualize
- Closely related organisms have similar restriction pattern
- Clustering of related organisms possible based on band pattern
- Restriction enzymes depend on the microorganism

Mainly for strain typing

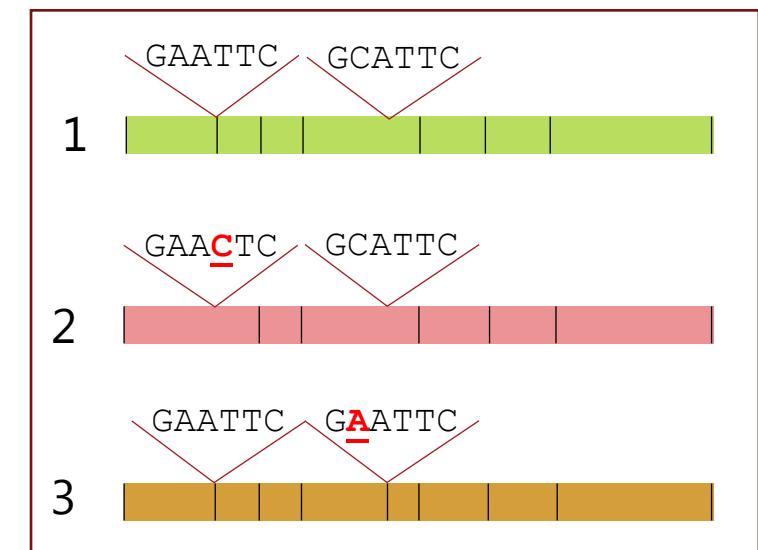
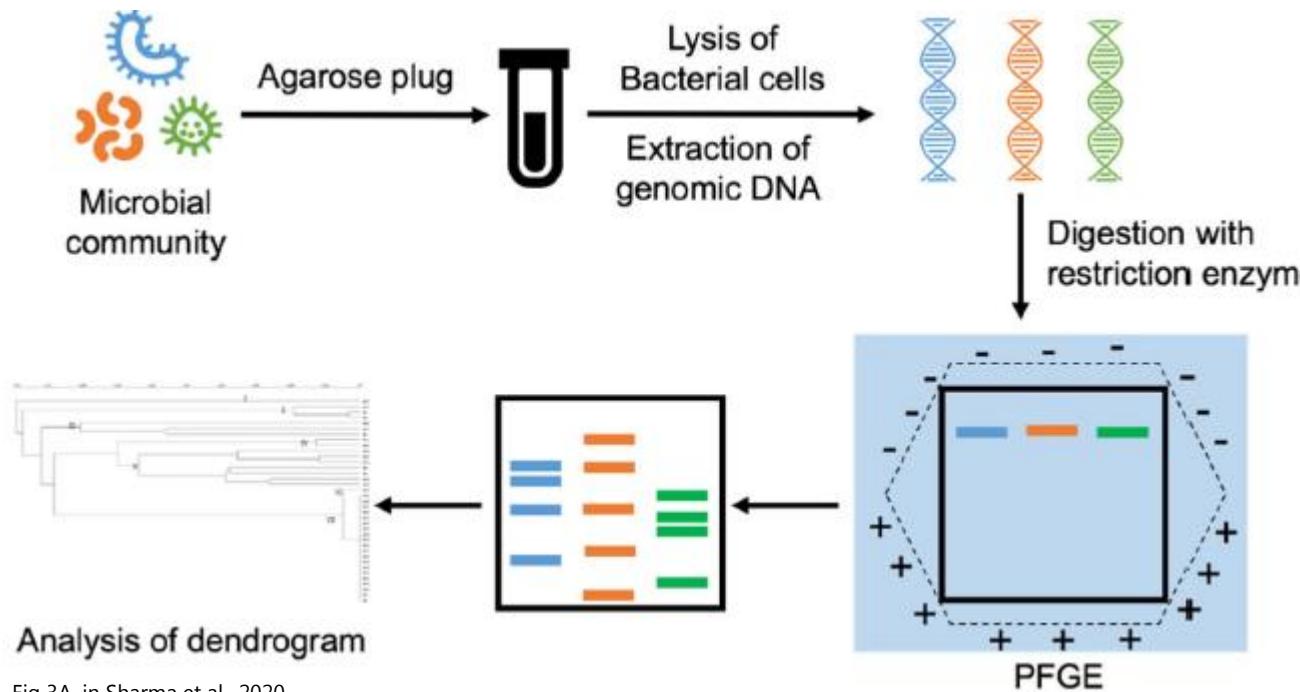
Pulsed field gel electrophoresis (PFGE) typing

- Chromosomes (e.g. **yeast** chromosomes) up to 10.000.000 bp (10 Mb) can be separated undigested
- **Bacterial** chromosomes (2-5 Mb) isolated and digested with rare cutting restriction enzymes can be separated and used for strain typing
- The chromosomal bands can be separated due to a switching electric current forcing the DNA segments to move in changing directions ("snake movement"). The switching interval is important for the success of the method and should be optimized for each experiment
- PFGE has been used for typing of microorganisms, establishing long range restriction maps, localization of specific genes etc.

PFGE

-Pulse field gel electrophoresis for bacteria (chromosome)

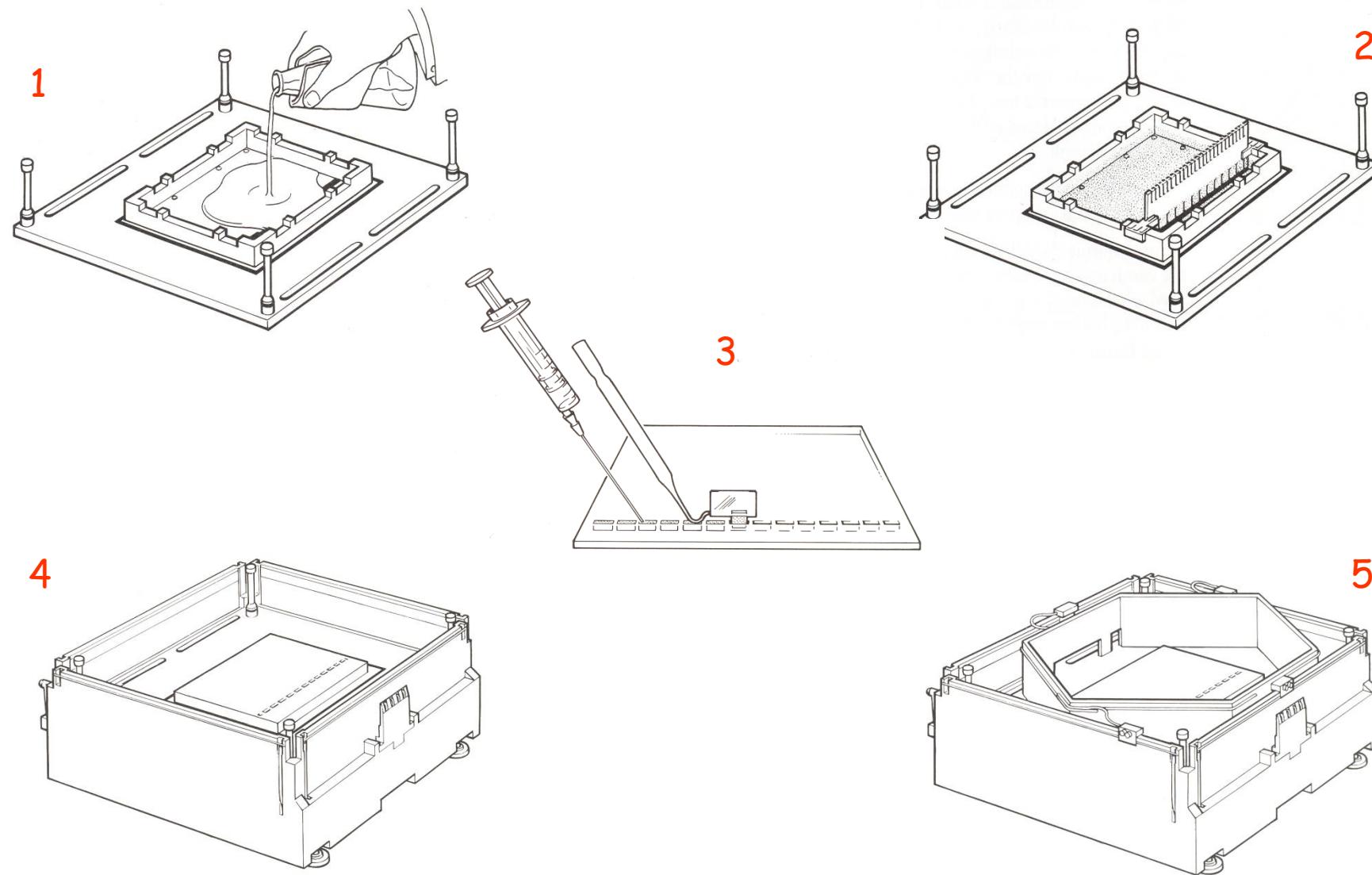
- Use restriction enzymes that digests seldom in the chromosome (<25-50 times)
- Switching the current facilitate separation of large DNA fragments



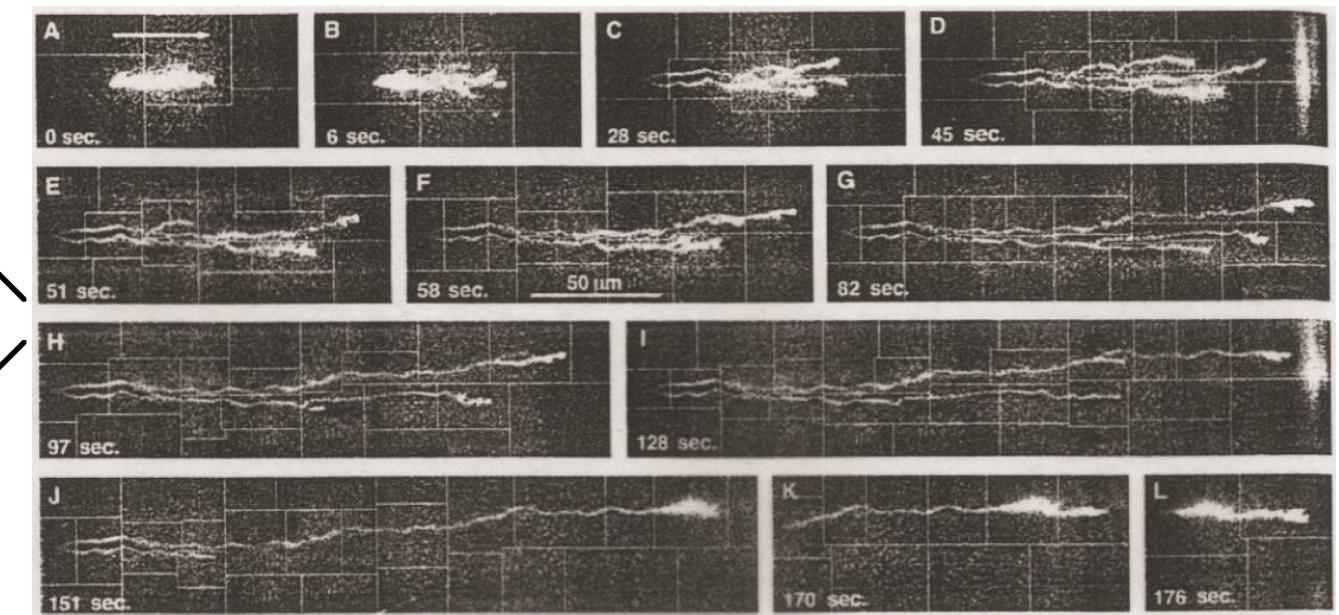
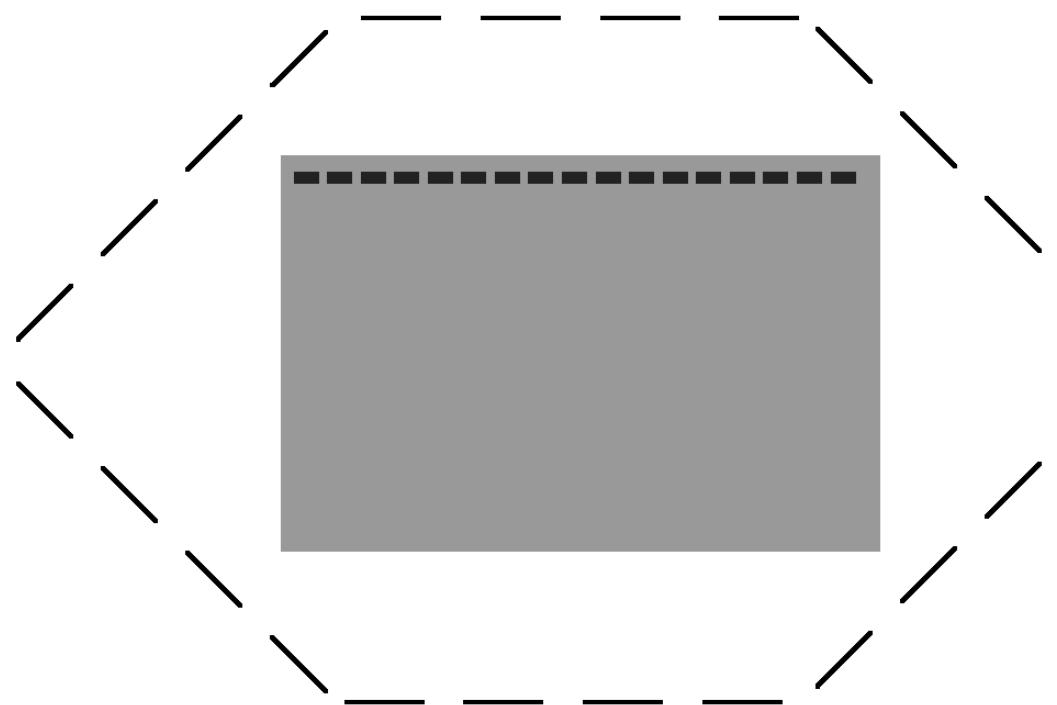
Protocol for typing of yeasts (PFGE)

- Propagation of the yeast (late exponential phase)
- Formation of spheroplasts
- Embedding of the spheroplasts in agarose blocks
- Protease treatment at 45°C over night
- Washing of the blocks and transfer to agarose gel
 - 165 V
 - 100-120 mA
 - Pulse (90 s for 14 h, 105 s for 12 h, 120 s for 14 h)
- Staining with ethidium bromide
- Visualisation of the chromosomes
- Documentation (photo or image analysis)
- Comparison with existing databases

PFGE



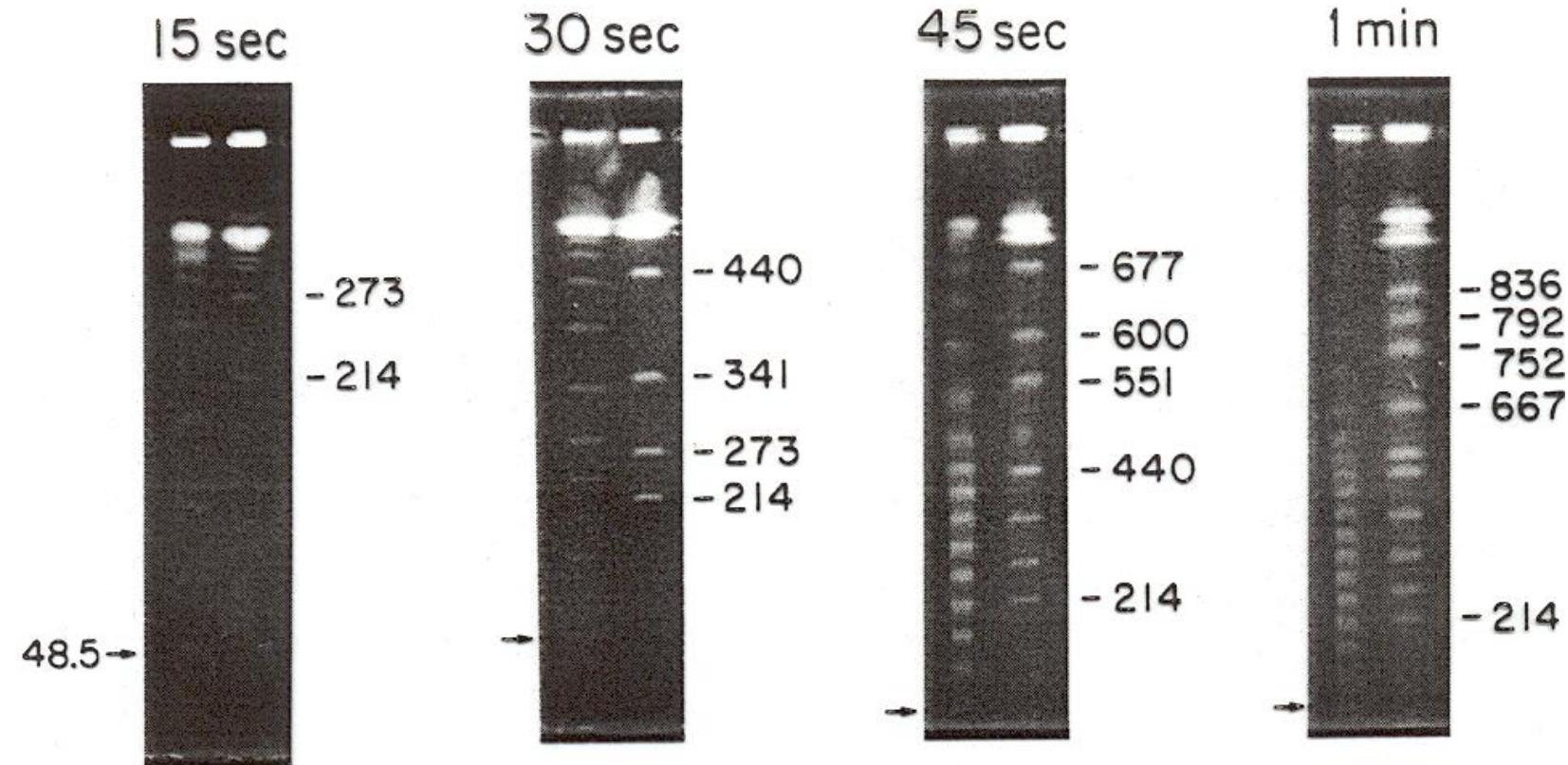
Pulsed field gel electrophoresis (PFGE) – used for separation of big DNA molecules



Running condition influence the movement of DNA through the gel

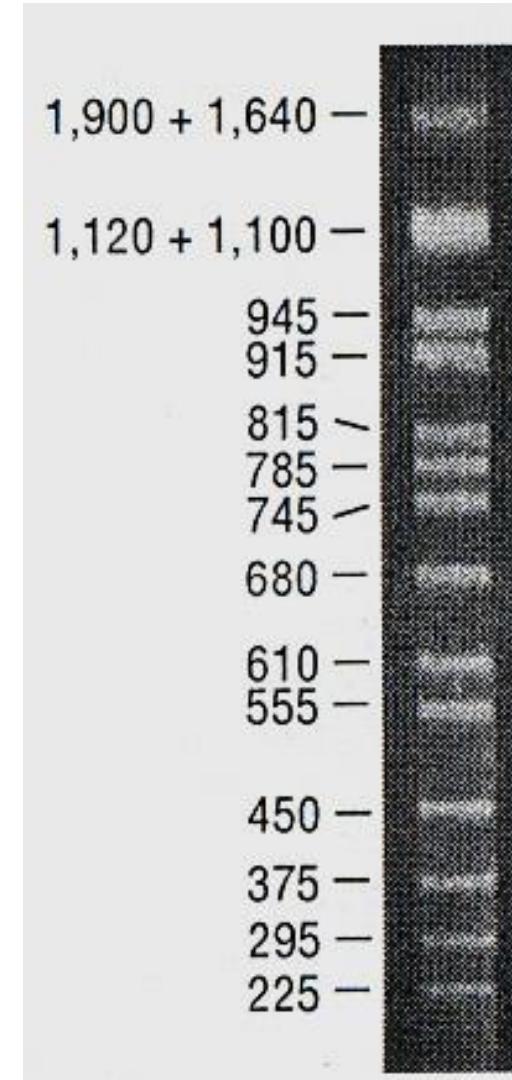
→ Switching electric current

PFGE resolution is influenced by switching interval



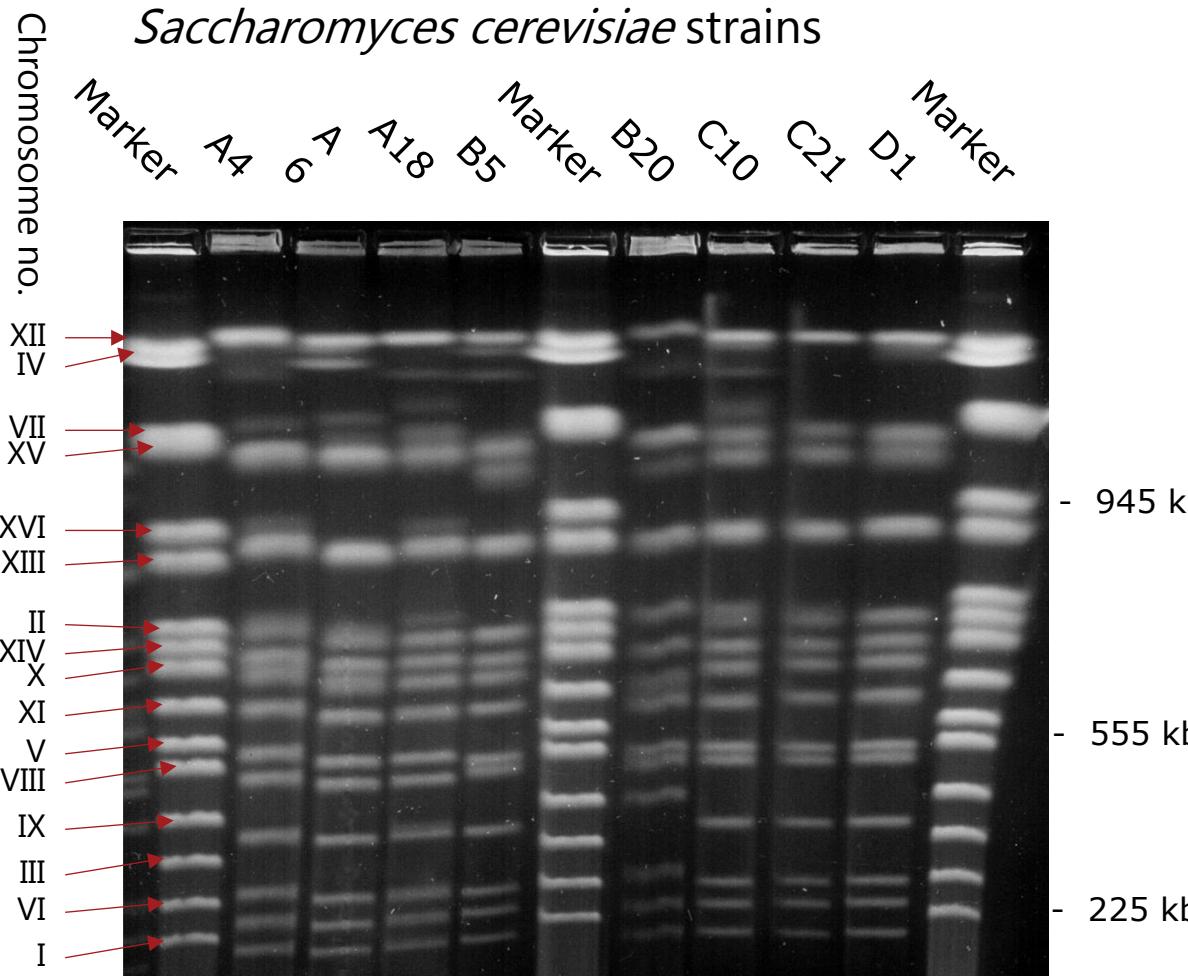
From: B. Birren & E. Lai Pulsed field Gel Electrophoresis: A Practical Guide. Academic Press, New York, 1993

Sizes of *Saccharomyces cerevisiae* chromosomes – PFGE marker



Chromosome	Size (kb)
XII	1900
IV	1640
VII	1120
XV	1100
XVI	945
XIII	915
II	815
XIV	785
X	745
XI	680
V	610
VIII	555
IX	450
III	375
VI	295
I	225

PFGE gel result for yeast and bacteria



Yeast might be haploid, diploid, tetraploid or aneuploid!

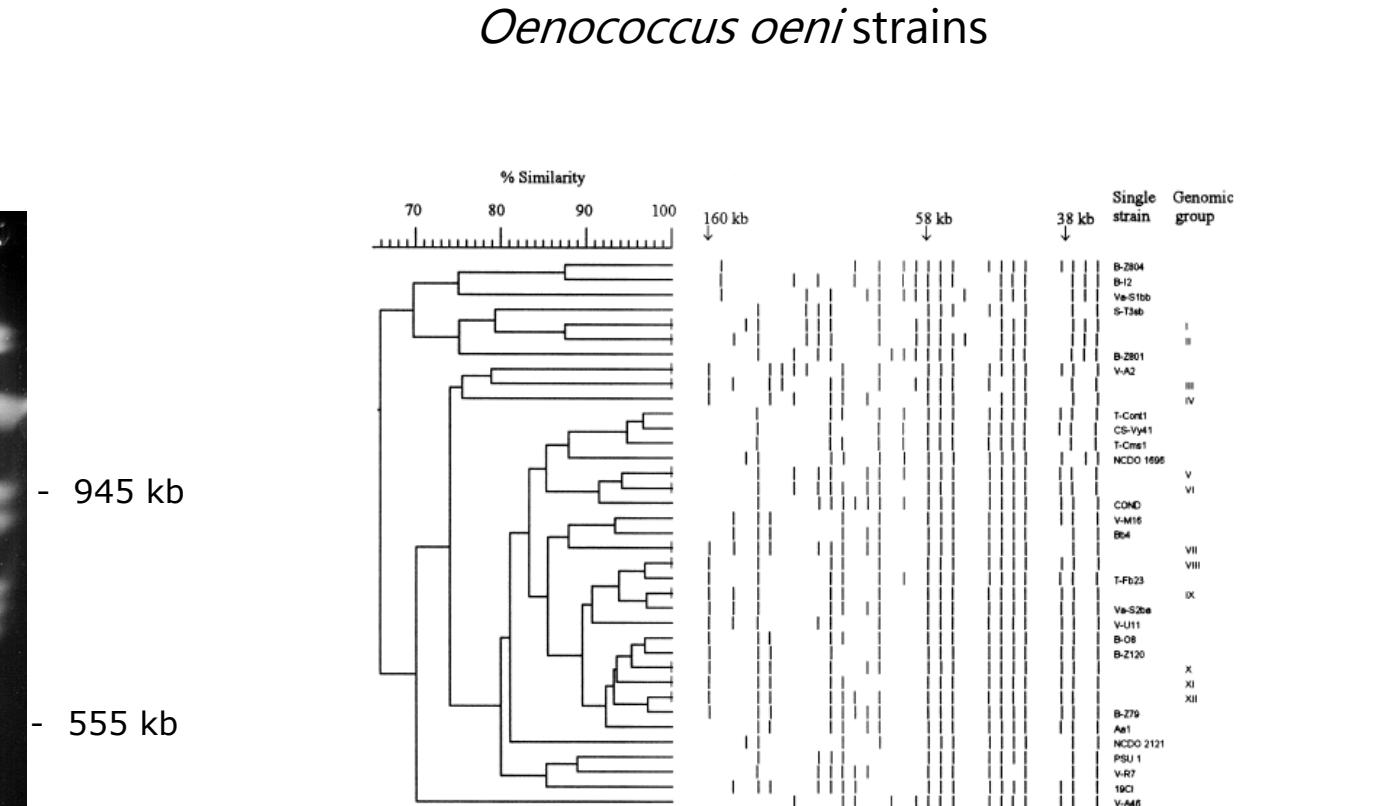


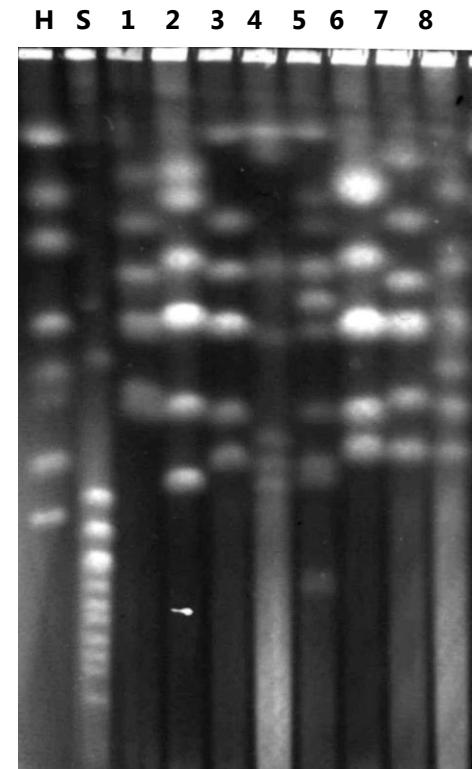
Fig. 1. UPGMA dendrogram based on the *Apa*I PFGE patterns of *Oenococcus oeni* strains. Roman numerals indicate groups of strains that have identical profiles (see Table 1 for details).

From: Zapparoli et al. (2000) Curr. Microbiol. 144, 351-355

PFGE estimated size of chromosomes – *D. hansenii*

Table 1 Estimated size of chromosomes and total genome size for strains of *Debaryomyces hansenii*

Strain	Origin	Size of chromosomal DNA (Mb)										Estimated
		1	2	3	4	5	6	7	8	9	10	
CBS117*	CBS	3.11	2.69	2.05	1.89	1.72	1.46	1.36	1.20			15.48
CBS164*	CBS	2.90	2.53	2.16	1.84	1.58	1.53					12.54
CBS766*	CBS	2.93	2.73	2.27	1.89	1.54	1.27					12.63
CBS767*†‡	CBS	3.14	2.56	2.20	1.85	1.51	1.36					12.62
CBS772*	CBS	3.14	3.05	2.22	1.78	1.43	1.32	1.24				14.18
CBS796*	CBS	2.76	2.57	1.98	1.75	1.68	1.34					12.08
CBS1102*	CBS	3.14	2.75	2.52	2.21	2.01	1.81	1.51	1.33	1.27	0.71	19.26
CBS1792*	CBS	2.83	2.30	1.86	1.52	1.40						9.91
CBS1800*	CBS	3.04	2.58	2.14	1.86	1.56	1.39					12.57
CBS8416*	CBS	3.03	2.81	2.24	1.89	1.82	1.67	1.54	1.38			16.38
CBS789§‡	CBS	2.68	2.43	2.08	1.69	1.46	1.24	0.31				11.89
CBS1796§	CBS	2.75	2.31	2.07	1.28	1.21	0.76					10.38
CBS4373§	CBS	2.81	2.50	2.06	1.68	1.52	1.25					11.82
CBS5230§	CBS	2.71	2.36	1.96	1.70	1.49	1.22					11.44
CBS5572§	CBS	2.80	2.20	1.97	1.53	1.44	1.24	1.08				12.26
CBS6066§	CBS	2.84	2.59	2.18	1.98	1.88	1.72	1.53	1.23			15.95
CBS7254§	CBS	2.57	2.39	2.06	1.71	1.49	1.20					11.42
CBS7761§	CBS	2.88	2.37	1.94	1.52	1.33	1.13					11.17
CBS7784§	CBS	2.23	1.92	1.51	1.40	1.23	1.10					9.39
CBS8417§	CBS	3.06	2.14	1.85	1.59	1.31	1.00	0.87	0.39			12.21
DI1*	Dairy A, smear	2.72	2.38	2.03	1.89	1.65	1.41	1.02				13.10
DI2*	Dairy A, smear	3.13	2.45	2.02	1.74	1.43	1.15					11.92
DI3*	Dairy A, smear	2.88	2.43	2.04	1.76	1.51	1.39					12.01
DI4*	Dairy B, smear	2.96	2.48	2.09	1.74	1.55	0.88					11.70
DI5*	Dairy A, smear	3.11	2.69	2.18	1.98	1.87	1.65	1.33	1.18	1.03		17.02
DI6*	Dairy C, smear	3.12	2.69	2.38	2.06	1.91	1.66	1.42	1.33	0.97		17.54
DI7*	Dairy C, smear	2.69	2.03	1.88	1.63	1.52	1.35	1.04				12.14



Journal of Applied Microbiology 2004, 97, 205–213

doi:10.1111/j.1365-2672.2004.02293.x

Genetic diversity of the species *Debaryomyces hansenii* and the use of chromosome polymorphism for typing of strains isolated from surface-ripened cheeses

K.M. Petersen and L. Jespersen

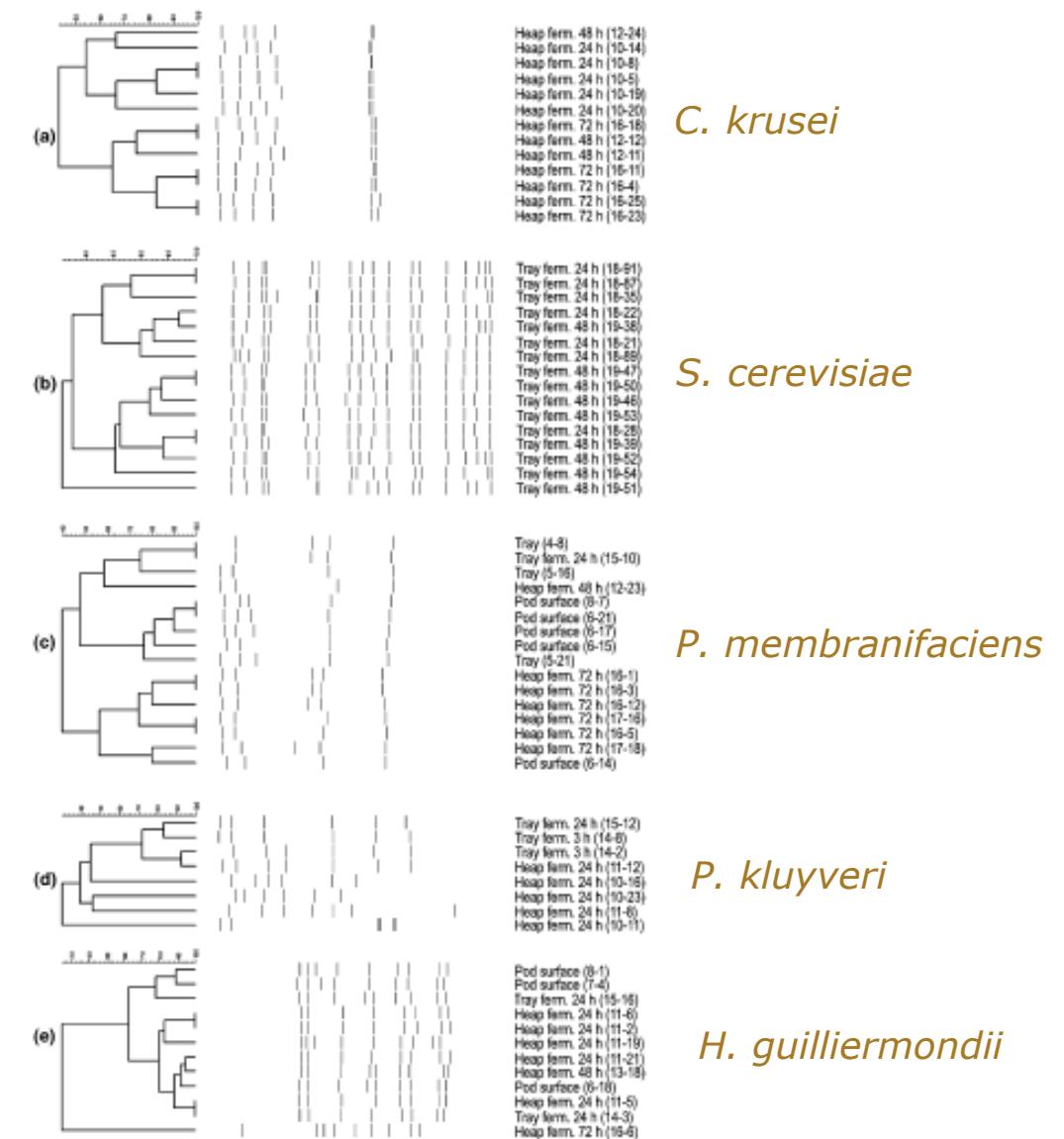
Department of Dairy and Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Frederiksberg C, Denmark

PFGE for typing to strain level

PFGE is the golden standard for typing to strain level !!!

The interpretation of PFGE results for yeasts is influenced by their chromosomal structure:

- Number of chromosomes vary between different yeast species
- Remember: yeast might be haploid, diploid, tetraploid or aneuploid!



FEMS Yeast Research 5 (2005) 441–453

FEMS
Yeast Research
www.fems-microbiology.org

Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans

Lene Jespersen *, Dennis S. Nielsen, Susanne Honholt, Mogens Jakobsen

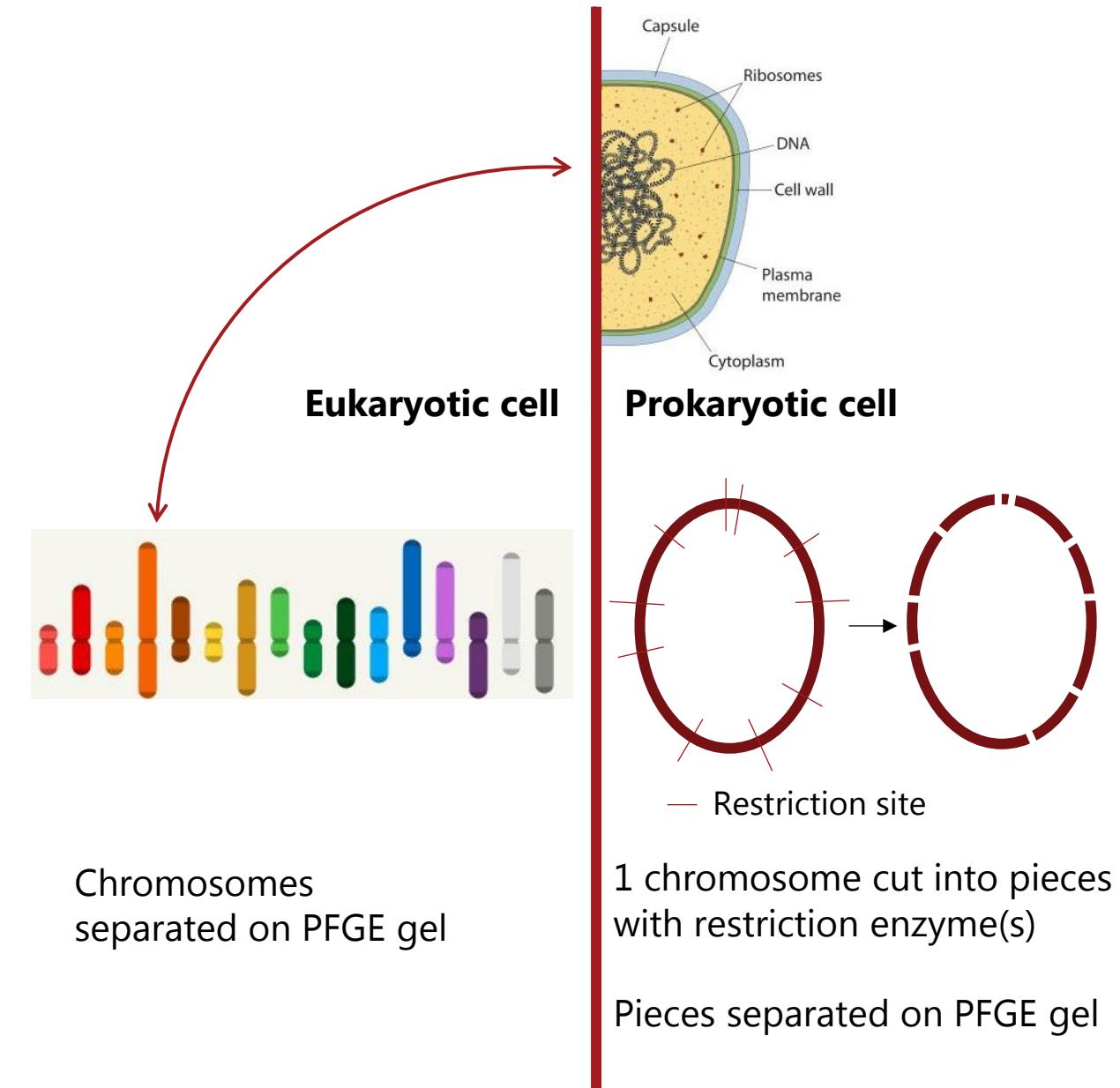
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PFGE

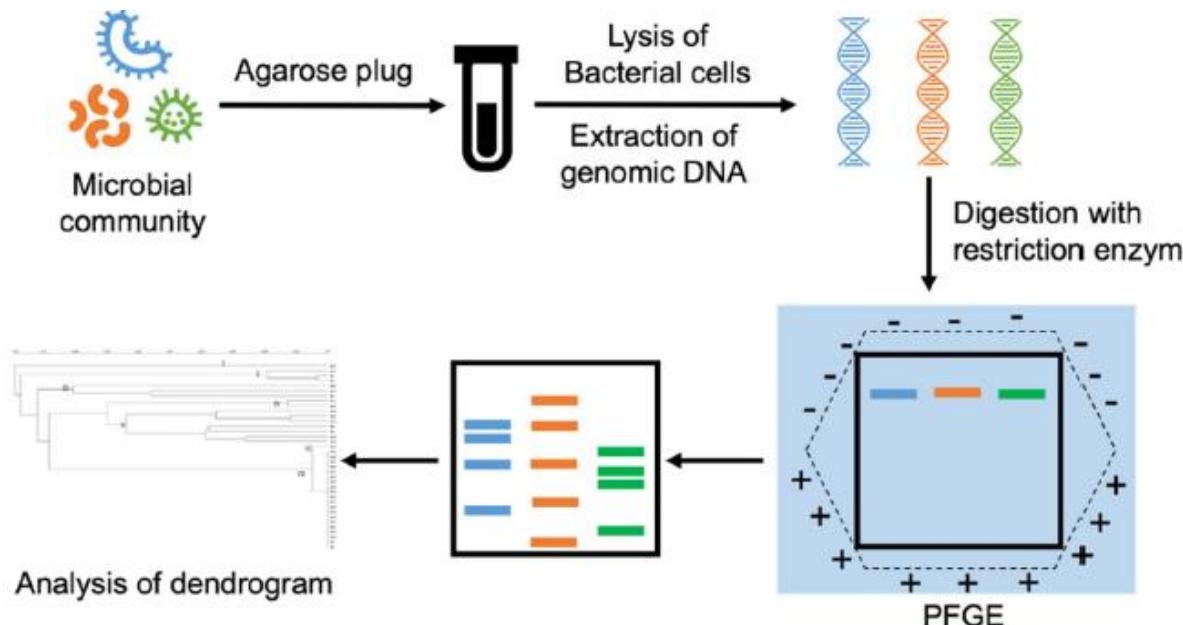
- Generate a fingerprint
- Resolution to strain level
- Golden standard for bacteria and yeast typing
- Laborious. Takes 3-4 days → often used when strain differences are investigated
- Different approach for bacteria and yeast!



Group work

Discuss with your neighbour for 3-5 minutes:

- When you do PFGE on yeast, what step is different from the PFGE flow chart for bacteria as shown below (Sharma et al, 2020) ?
- How many bands would you find on a PFGE gel for the yeast and the bacteria below?



For yeast the number of bands may vary slightly dependent on whether all chromosomes are separated and chromosomal structures as e.g. aneuploidy

Fig 3A, in Sharma et al., 2020

MLST

-multilocus sequence typing

- PCR amplification of part of 5-10 genes encoding metabolic house-keeping proteins
- Sanger sequencing of amplified genes
- Strains recognized based on "single nucleotide polymorphisms" (SNP's)
→ allele → Sequence Type (ST)
- Databases:
 - <http://pubmlst.org/>
~129 microbial species represented

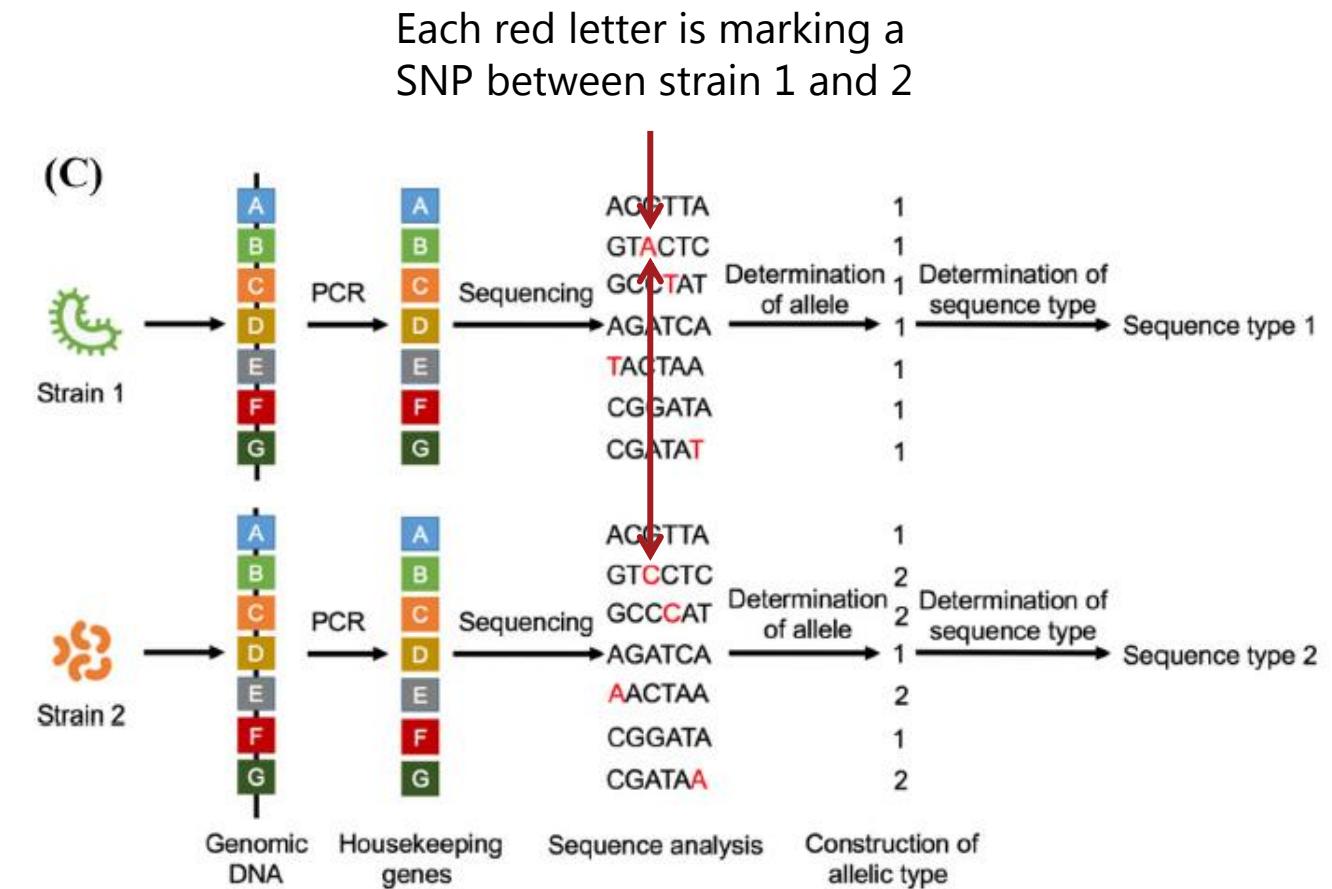


Fig 4C, in Sharma et al., 2020

Mainly for strain typing

MLSA: -multilocus sequence analysis

- Used for identification between a broad number of genera
- Translated DNA sequences (protein sequence) of highly conserved genes found in all bacteria are aligned, and clustered
- Typically sequences from 2-3 gene products used:
 - *gyrB, recA, rpoB, rpoA, pheS, atpA*, (Bacteria)
 - Can reach species or subspecies level
- Used to generate phylogenetic trees and subsequently deduce phylogenies. Could replace DNA–DNA hybridization

Zhu et al., *Int J Syst Evol Microbiol* 2018;68:1672–1677

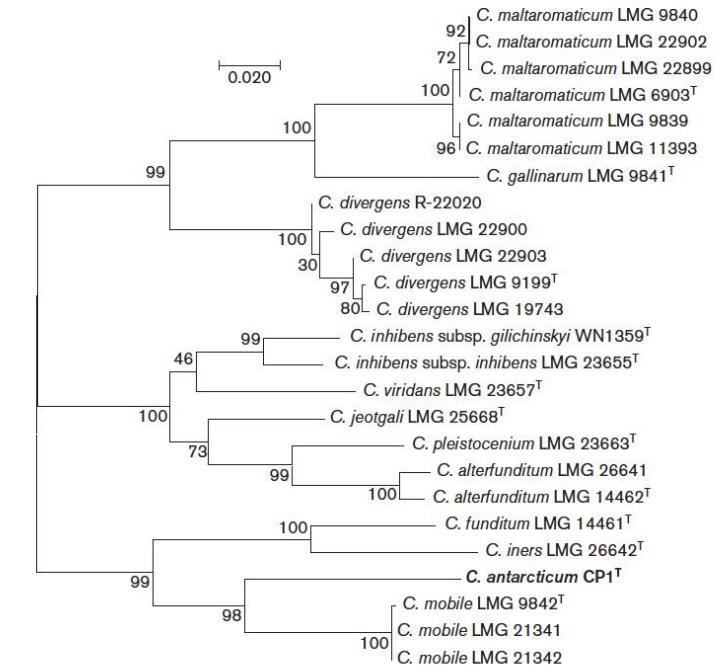
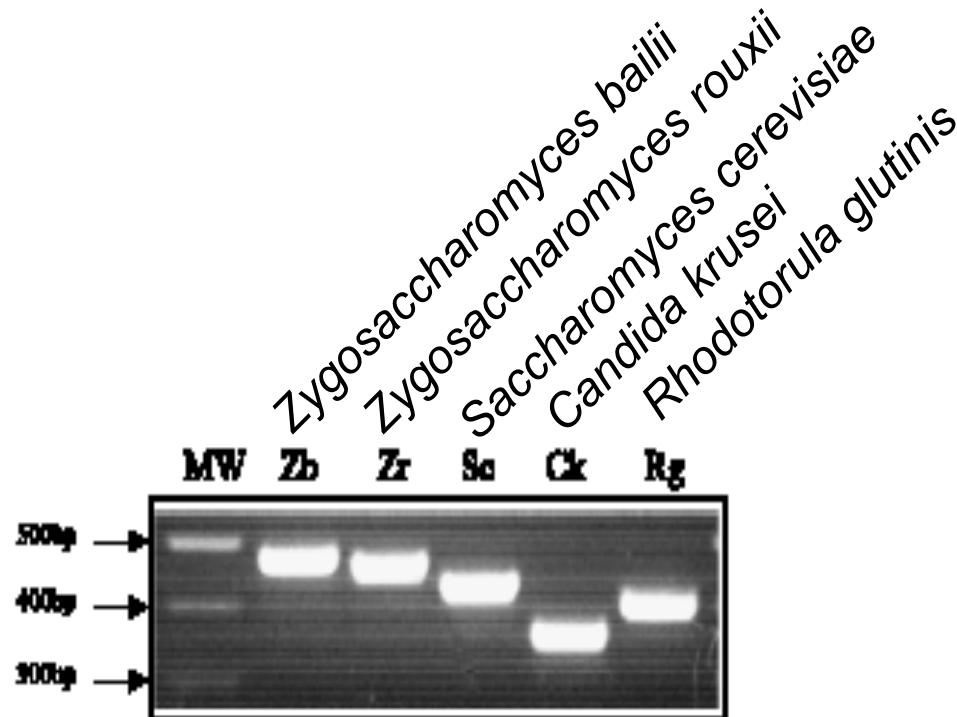


Fig. 2. Molecular phylogenetic analysis based on concatenated *pheS*, *rpoA* and *atpA* sequences (982 bp) of strain CP1^T and other species of the *Carnobacterium* genus reported previously using the maximum-likelihood method based on Kimura's two-parameter model in MEGA 7.0. Bootstrap values based on 1000 replications are shown at branch nodes. Bar, 0.02 Knuc units.

Species specific PCR

- Primers designed to target specific sequences unique to the target microorganism
- Amplify target sequence using PCR
- Visualise on e.g. agarose gel
- Used routinely in diagnostic laboratories for detection of pathogenic fungi – e.g. *Candida albicans* and *Candida glabrata* – and spoilage yeasts e.g.: *Dekkera bruxellensis* (wine spoilage)

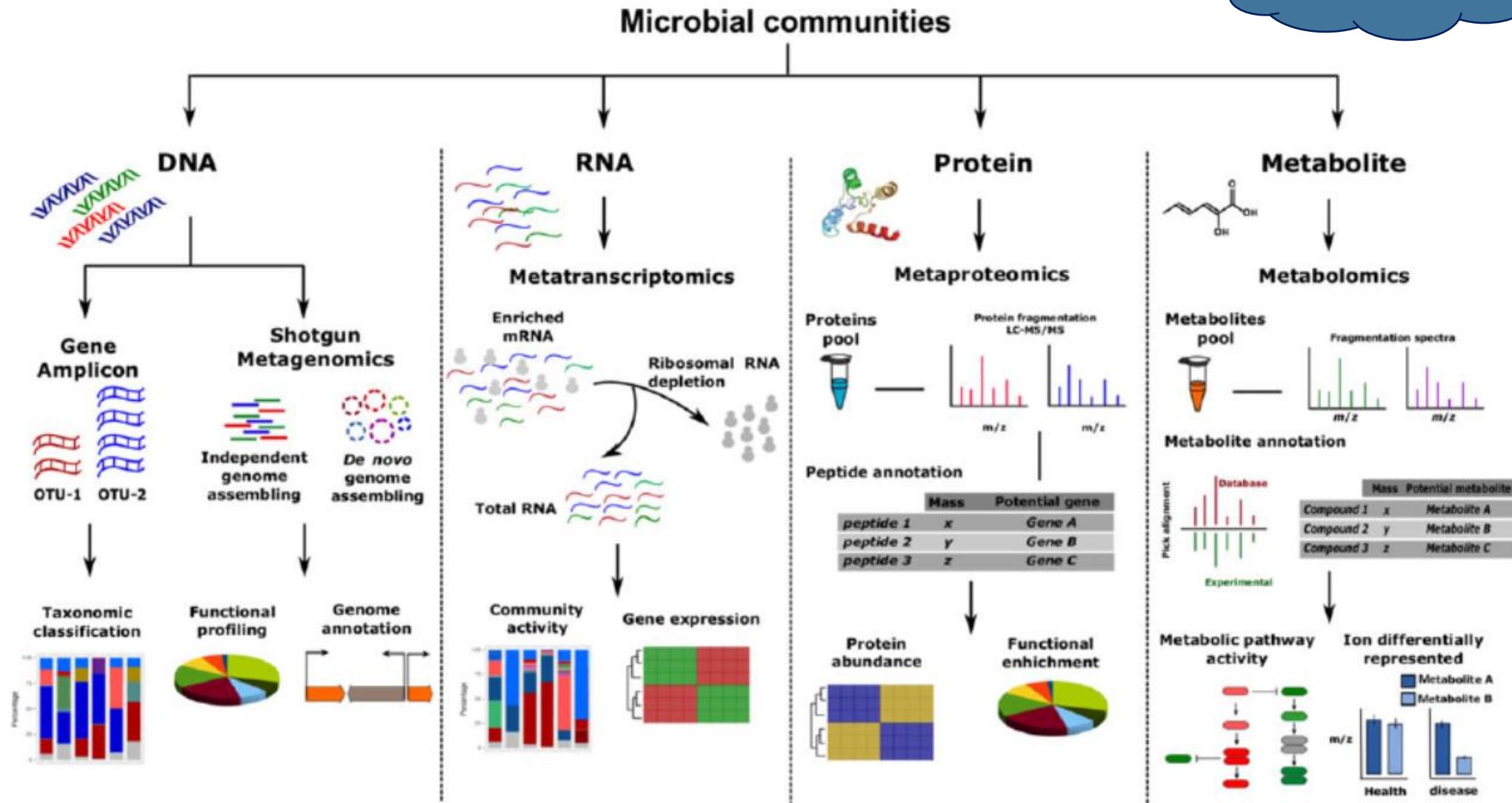


Cassey & Dobson (2004) IJFM 91:327-335

Mainly for species identification

Genomics, proteomics and metabolomics

Explained in a
later lectures

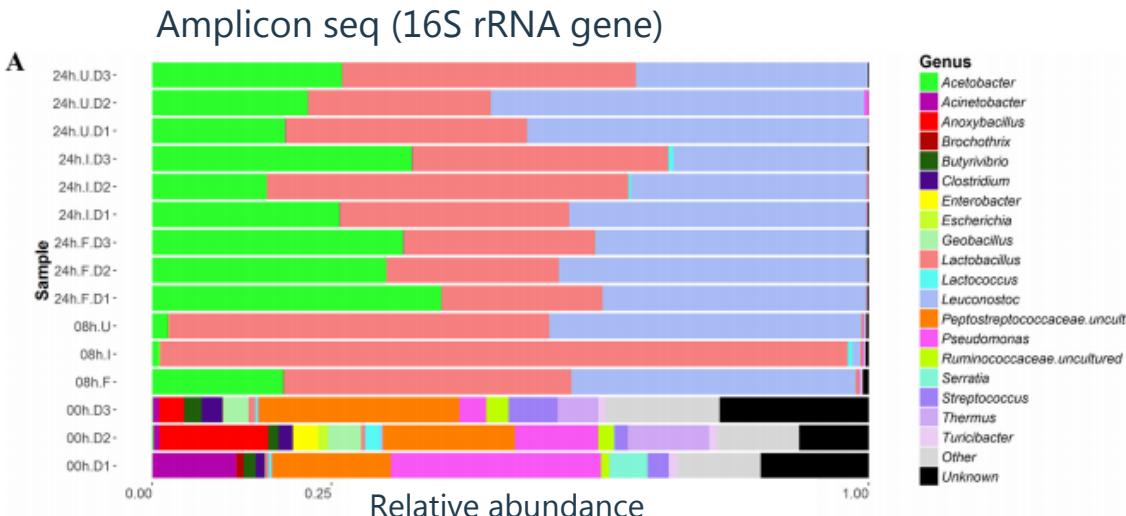


Metagenomics sequencing (total DNA)

Explained in a later lectures

High throughput amplicon sequencing based methods (16S rRNA gene for bacteria, ITS for yeast/fungi) allowing the detection of abundances of species (both alive and dead cells are detected) in microbial consortia.

- Relative quantification “who is there and in what fraction”
- Resolution sometimes limited (genus/species level)
- The difficult/tedious part is the bioinformatics, not the sequencing



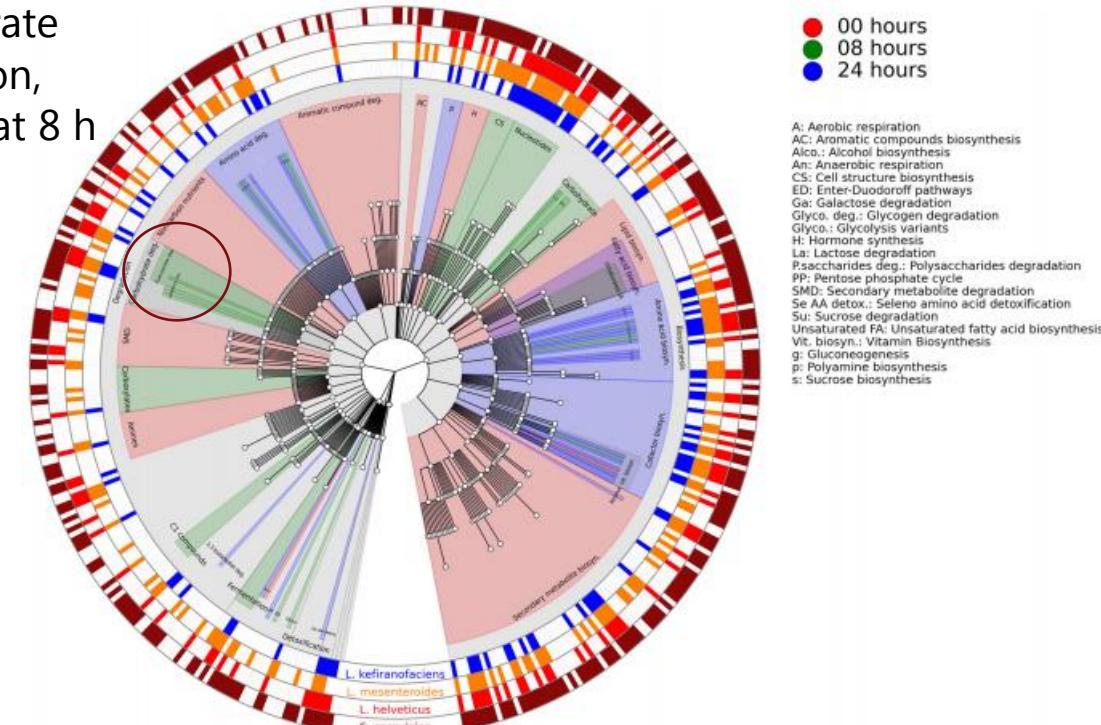
Mainly used for detection of the abundances of species (alive and dead cells are detected when DNA is sequenced!)

Amplicon and whole-metagenome shot gun sequencing

- Kefir grains from France, the UK and Ireland
- Sampled after 0, 8 and 24 h

Whole-metagenome shot gun seq
What genes are found?

Carbohydrate
degradation,
prevalent at 8 h



Walsh et al., 2016, mSystems 1(5):e00052-16

Only used for detection of genes

Sum up with your neighbour (2-3 persons) - 5 minutes

- Each identification method has strengths and limitations
 - Why do culture-**dependent**?
 - Why do culture-**independent**?
 - Why not?
 - Why not?

Sum up

- Each identification method has strengths and limitations as listed in the table
- Why do culture-**dependent**?
 - Phenotypic information
 - Technological properties can be investigated
 - Equipment available in most labs
- Why not?
 - Laborious and time consuming
 - Viable but not cultivable microorganisms cannot be detected
- Why do culture-**independent**?
 - You don't have to culture/grow the microorganisms
 - Viable but not cultivable microorganisms can be detected
 - Rapid (sometimes...)
- Why not?
 - No phenotypic information
 - No knowledge about technological properties
 - Price (equipment)
 - Mostly relative quantifications

Key messages

- ✓ Identification describes the “naming” of microorganisms at the species level.
- ✓ Typing describes the characterisation at the strain level.
- ✓ Identification methods with isolation steps gives the possibility for growing the microorganisms and determining their technological properties.
- ✓ Prokaryotes are predominantly identified by sequencing of the 16S rRNA gene and eukaryotes by sequencing of the 26S rRNA gene or parts of the ITS region.
- ✓ MALDI-TOF MS and FTIR can be used for identification. However, the methodologies (especially FTIR) might be influenced by growth conditions and the databases available.
- ✓ Techniques such as rep-PCR can be used for grouping of large amounts of isolates
- ✓ Several molecular typing techniques exist, but their applicability varies. Some of the typing techniques will also partly reveal the identification of the species e.g. if “type strains” are used as reference.
- ✓ Metagenomic approaches are increasingly used for characterisation of microbial consortia in foods.

Moulds and their role in fermented food and beverages

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Intended leaning outcome:

- ✓ You should be able to know the basic technological properties of moulds for use in the food and beverage industry
- ✓ You should be able to explain the basic of moulds taxonomy
- ✓ You should have a broad knowledge on the use of moulds in various products around the world and to be able to make idea generation on new innovative products

Background:

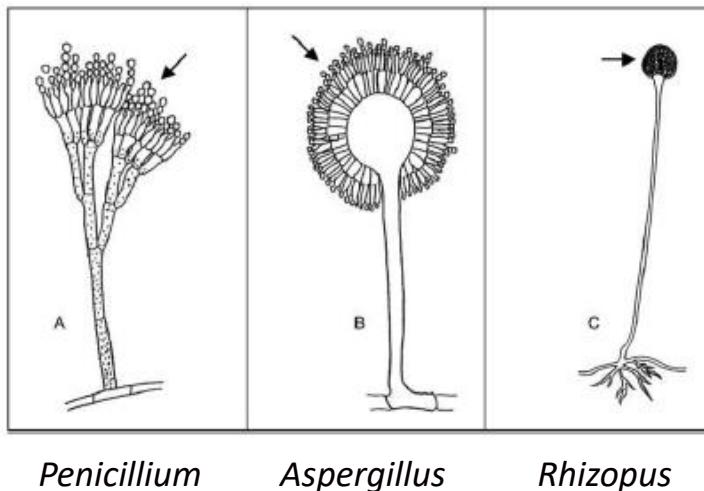
- ✓ Moulds are used in a number of food and beverages globally.
- ✓ Moulds are excellent enzymes producers and therefore well suited for raw materials which need extensive degradation.
- ✓ Moulds do offer a palette of different functionalities such as e.g. structural properties, colour, flavour characteristics etc.
- ✓ Moulds also have some significant drawbacks due to their oxygen requirement and their potential production of mycotoxins



Moulds - snapshot

- Eukaryotic multicellular organisms
- Composed by filaments called hyphae
- Moulds produce spores, when imperfect they are referred to as conidia

Ex: *Penicillium roqueforti* and *Penicillium camemberti* produce conidia
Rhizopus and *Mucor* produce spores (in sporangia)

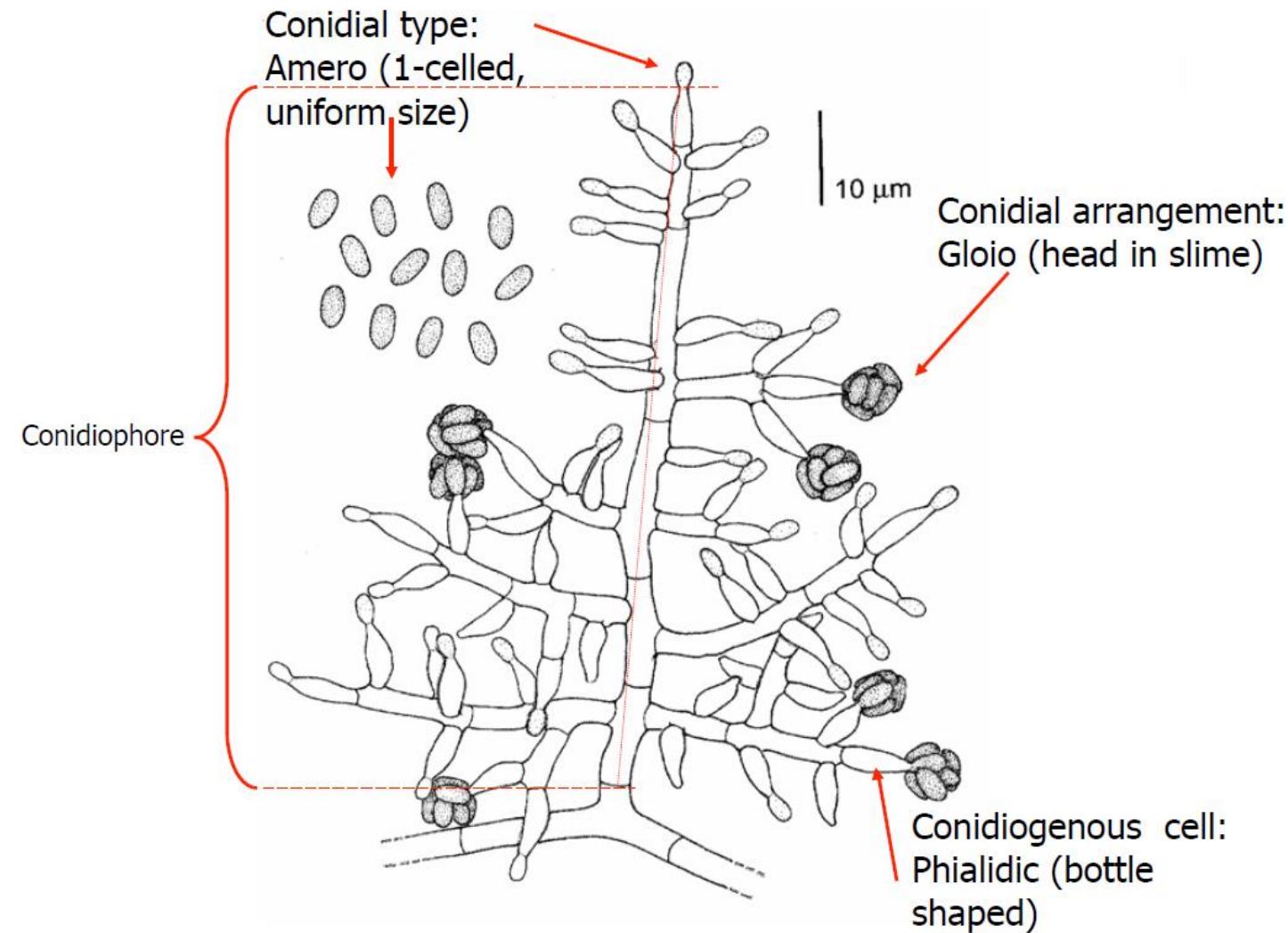


Roquefort
cheese

Rice koji

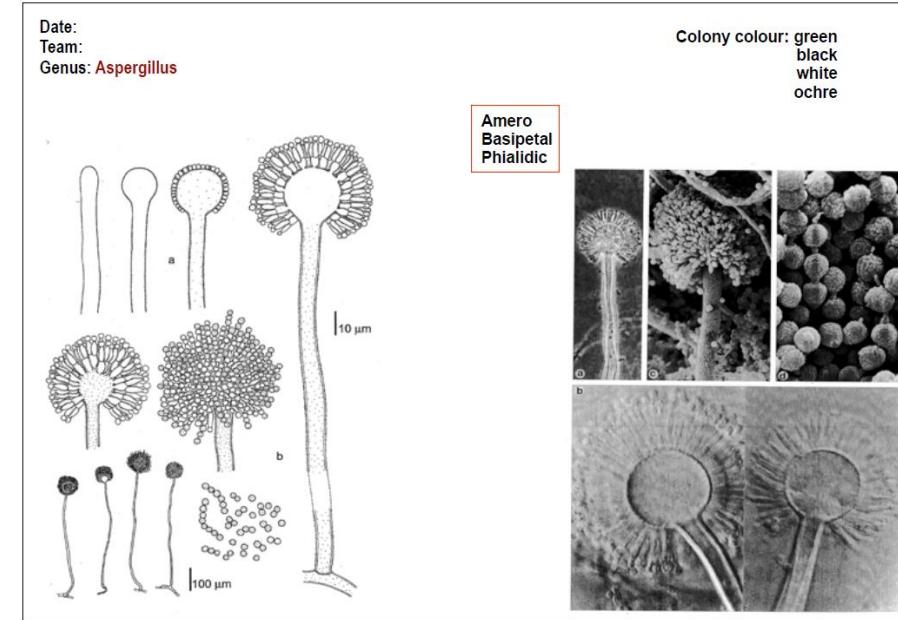
Tempeh

Identification based on the micro-morphology of conidia

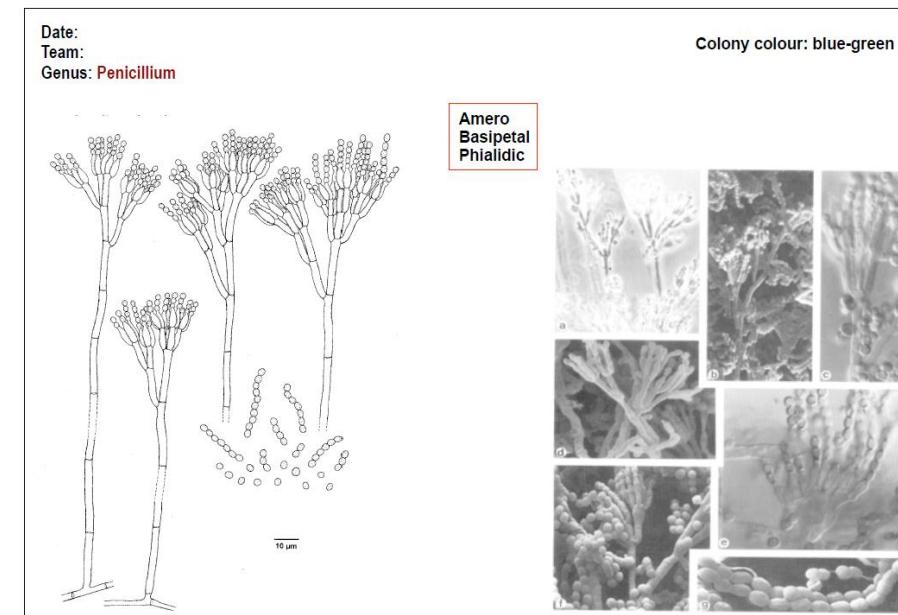


Moulds in fermented food and beverages - examples (1/2)

Aspergillus spp.

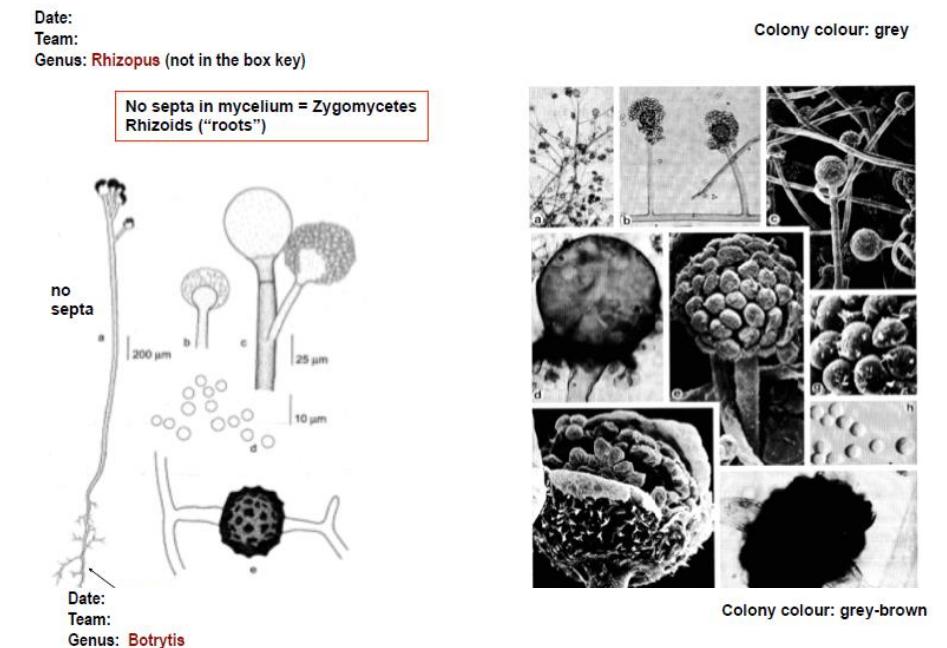


Penicillium spp.

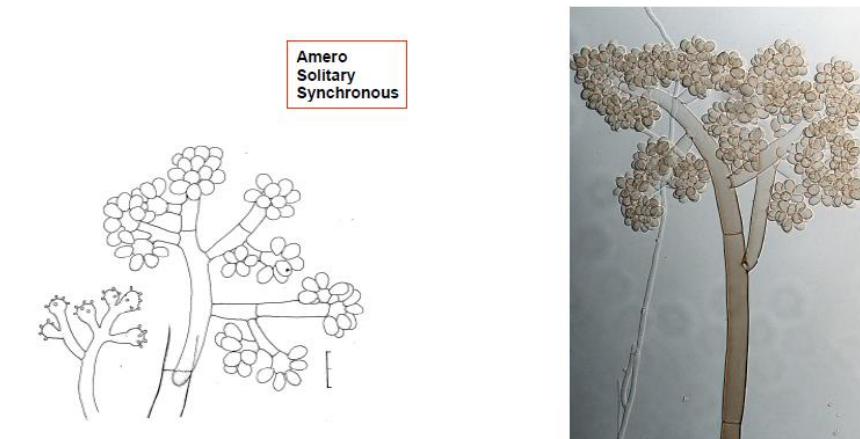


Moulds in fermented food and beverages - examples (2/2)

Rhizopus spp.



Botrytis spp.



Identification of moulds

- ✓ Metabolite profile, e.g. mycotoxins and enzymes
- ✓ Physiological properties
- ✓ Resistance
- ✓ Ecological properties
- ✓ Morphology and life cycles
- ✓ Genetic machinery
- ✓ ...



...it is important to identify
the fungi at **species** level

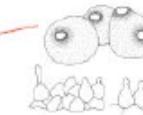
Identification by micro-morphology

"either-or" key

KEY TO THE COMMON FOOD- AND INDOOR ANAMORPHIC GENERA

1a. Conidia borne in closed structures; pycnidia.....

Phoma (p.310)



1b. Conidia borne on open structures; on hyphae, conidiophores, sporodochia or synnemata.....

2

2a. Conidia one or two-celled produced in chains.....

3

2b. Conidia produced in slime heads.....

10

2c. Conidia produced solitary.....

14

3a. Chains formed via basipetal succession.....

4

3b. Chains formed via acropetal succession.....

19

3c. Chains formed via thallic succession.....

21

4a. Conidia usually two-celled; retrogressive basipetal succession, arranged like a spike; colonies pinkish.....

Trichothecium (p.332)

4b. Conidia one-celled; basipetal succession

5

5a. Conidia produced from specialized conidiogenous cells; annellides; conidia truncate.....

6

5b. Conidia produced from specialized conidiogenous cells; phialides; conidia globose to fusiform

7

6a. Synnemata absent.....

Scopulariopsis (p.312)

6b. Synnemata present, dark grey to black

Doratomyces (p.170)

7a. Conidiophores with apical swelling.....

Aspergillus (p.104)

7b. Conidiophores without apical swelling

8

8a. Conidia and conidiophores dematiaceous; colonies blackish green.....

Stachybotrys echinotricha (p.324)

8b. Conidia and conidiophores hyaline; colonies white, yellow, green or brown

9

9a. Phialides with short neck; colonies white or various shades of green.....

Pericillium (p.206)

9b. Phialides with long neck; colonies yellow to brown

Paeciliomyces (p.202)

11

10a. Conidia both one- and multi-celled; colonies white, yellow, pink or red

Fusarium (p.176)

10b. Conidia only one-celled

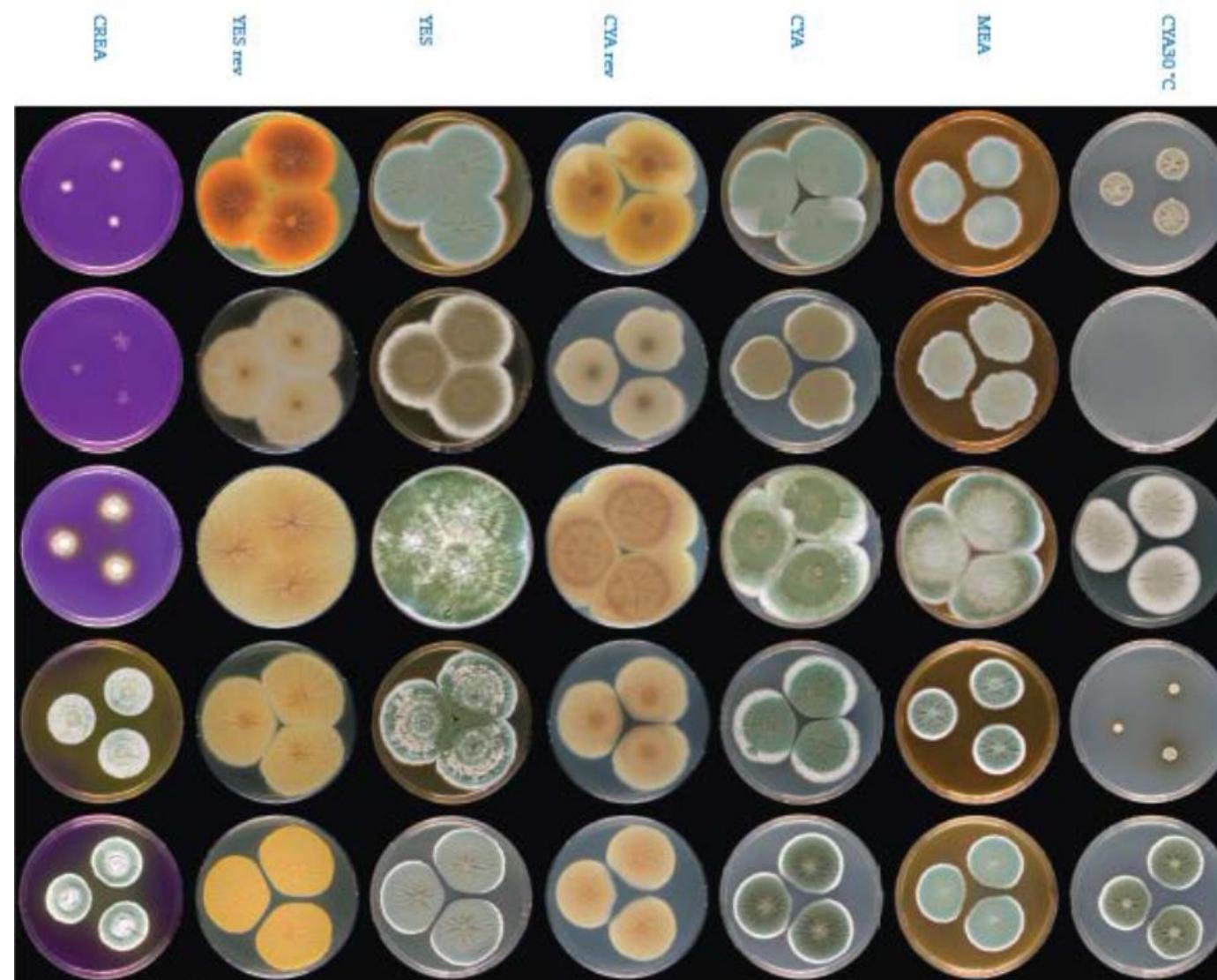
11

Conidia/spores are important taxonomic characteristics

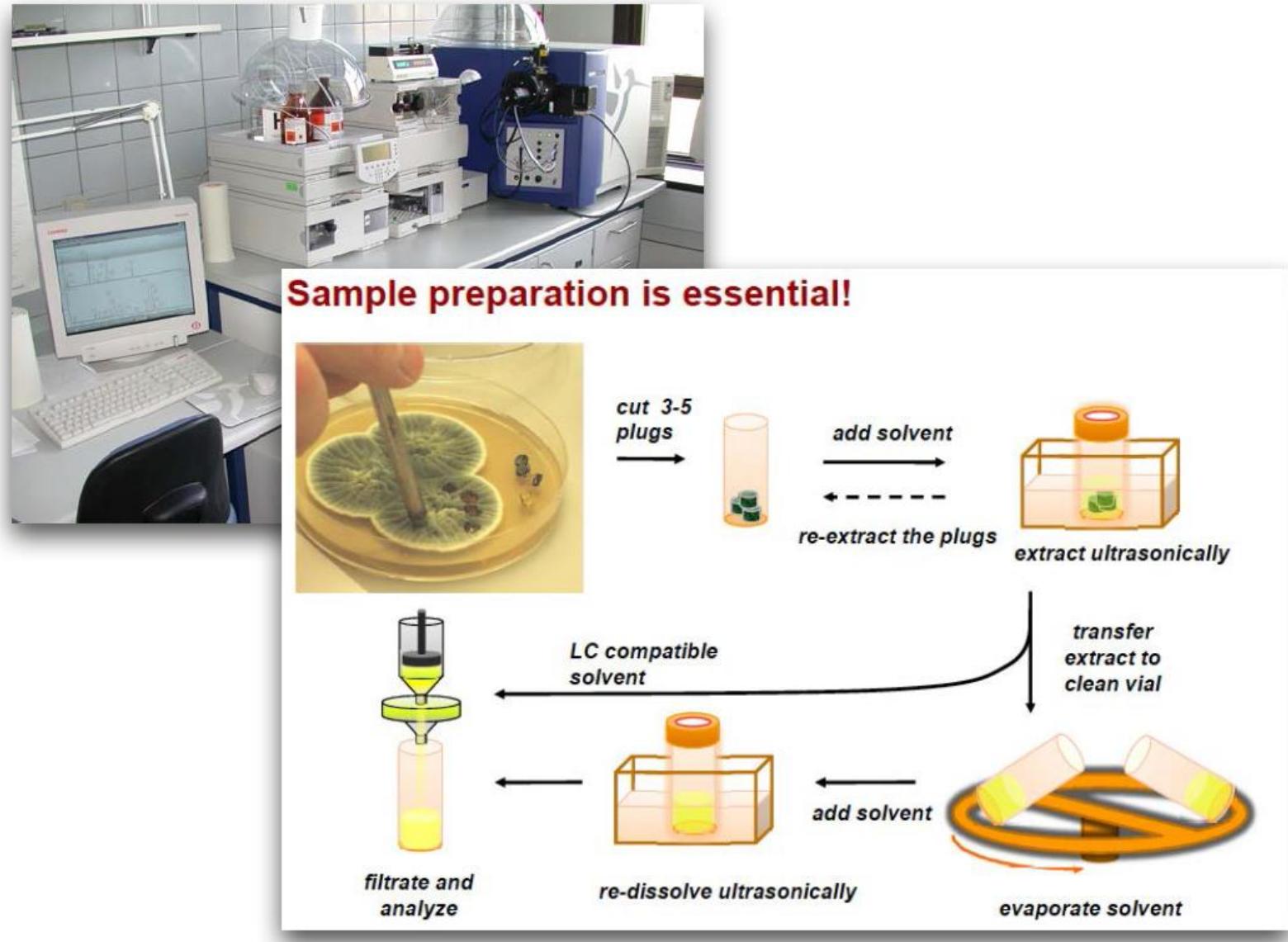
"Box key"

Conidia	Conidial arrangement	Conidiogenous cell	Colour	Genus	Colony colour and conidial characteristics
amero	pycnidal	phialidic	Pigmented/Hyaline	Phoma	Sparse mycelium with dark, oozing pycnidia, multi-cell. at any dia. pores.
				Chrysosporium	Pink, fast growing colonies, large cylindrical conidia > 6 μ .
				Geotrichum	White, yeast-like colonies, large barrel-shaped conidia > 6 μ .
	thallic	nonspecialized	Hyaline	Geomyces	Greyish, slow growing colonies, small barrel-shaped conidia < 6 μ .
				Oidiodendron	Greyish, slow growing colonies, small, ovoid conidia < 6 μ .
				Wallemia	Brown, slow growing colonies, dark, rough conidia.
	gloeo	phialidic	Hyaline	Fusarium	White, yellow or red colonies, also didymo- and/or phragmo-conidia.
				Acetmonium	White, slow growing colonies, long awl-shaped phialides.
				Phialophora	Grey to olive coloured, slow growing colonies, phialides with collarette.
basipetal	acropetal	phialidic	Hyaline	Trichoderma	Green, fast growing colonies, short phialides.
				Stachybotrys	Dark green to black colonies, black conidia.
				Aspergillus	Various coloured colonies, various growth speeds, conidiophore inflated at the apex.
	thallic	nonspecialized	Hyaline	Fusarium	White, yellow or red colonies, also didymo- and/or phragmo-conidia.
				Paeciliomyces	Lilac or yellow to brown colonies, long-necked phialides.
				Penicillium	White to various green colonies, flask-shaped phialides.
	gloeo	Pigmented	Hyaline	Stachybotrys	Dark green to black colonies, black conidia.
				Wallemia	Brown, slow growing colonies, dark, rough conidia.
				Scopulariopsis	White to brown, powdery colonies, truncate conidia.
acropetal	basipetal	nonspecialized	Hyaline	Doratomyces	Grey to black, slow growing colonies, truncate conidia on synnemata.
				Moniliella	Cream to brown colonies, dark chlamydospores.
				Cladosporium	Olive green colonies, also didymo- and/or phragmo-conidia.
	solitary	synchroous	Hyaline	Botrytis	Grey to brown colonies, fast growing, sometimes black sclerotia.
				Aureobasidium	Sparse mycelium, various coloured colonies, dark chlamydospores.
				Engyodontium	White, ricaceous colonies.
	acropetal	specialized	Hyaline	Triticozum	Pink to lilac, slow growing colonies.
				Monilia	Cream to brown colonies, dark chlamydospores.
				Cladosporium	Olive green colonies, also didymo- and/or phragmo-conidia.
dictyo	gloeo	acropetal	Hyaline	Trichothecium	Pink, fast growing colonies, conidia with truncate basal scar.
				Botryotrichum	White, yellow or red colonies, also didymo- and/or phragmo-conidia.
				Cladosporium	Olive green colonies, also amero- and/or phragmo-conidia.
	solitary	basipetal	Hyaline	Fusarium	White, yellow or red colonies, also amero- and/or didymo-conidia.
				Paeciliomyces	White, yellow or red colonies, also ovoid dictyo-conidia.
				Alternaria	White or various green colonies, also ovoid dictyo-conidia.
	acropetal	solitary	Hyaline	Curvularia	Brownish green colonies.
				Alternaria	White or various green colonies, also ovoid phragmo-conidia.
				Ulocladium	Dark green colonies, obovoid conidia.
phragmo	gloeo	acropetal	Hyaline	Stemphylium	Green colonies, infested with annellides with a dark band, dried out during dia.
				Epicoccum	White, orange or green colonies, conidia in sporodochia.
				Penicillium	White, yellow or red colonies, also didymo- and/or phragmo-conidia.

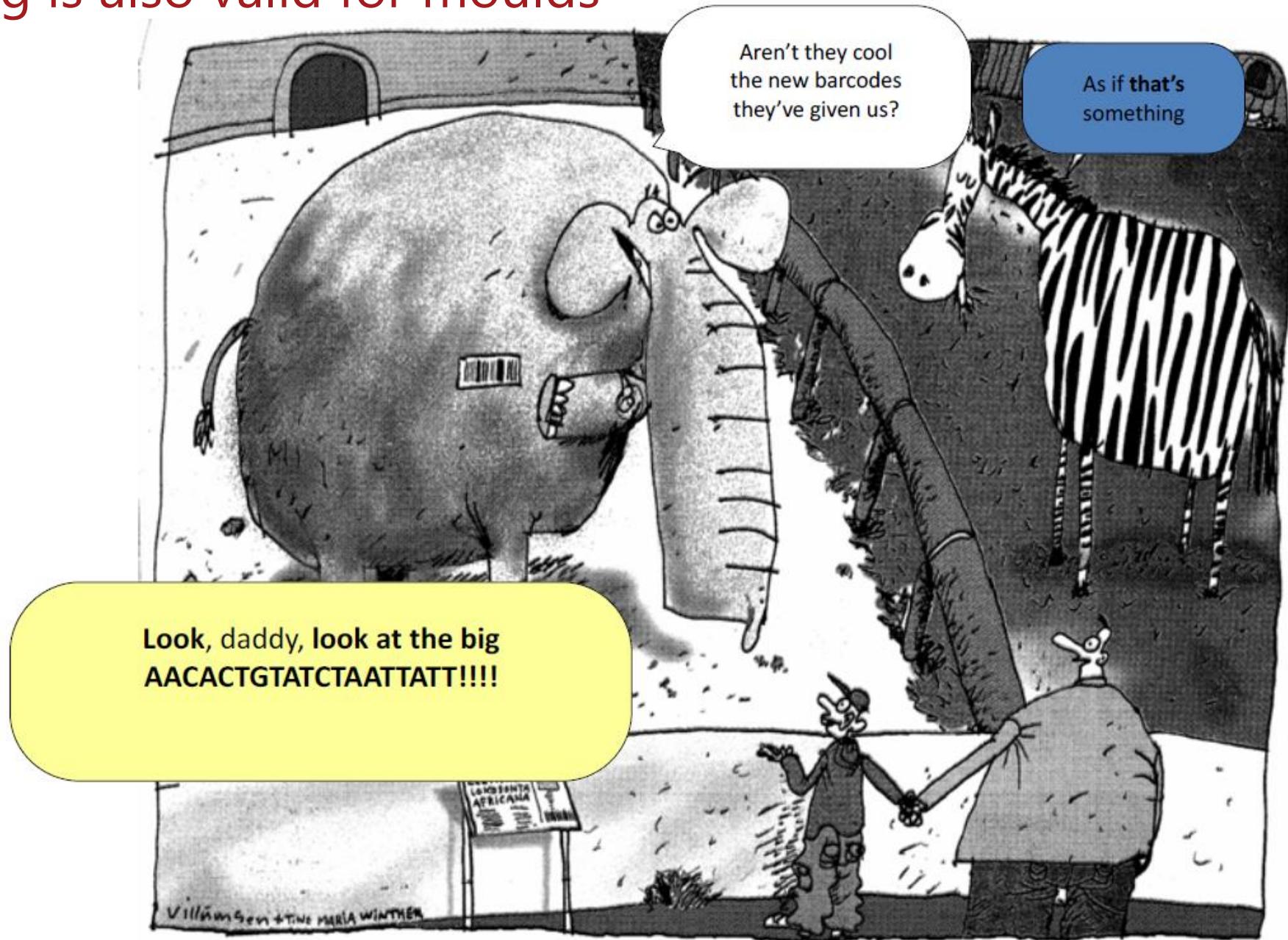
Identification by growth on specific media



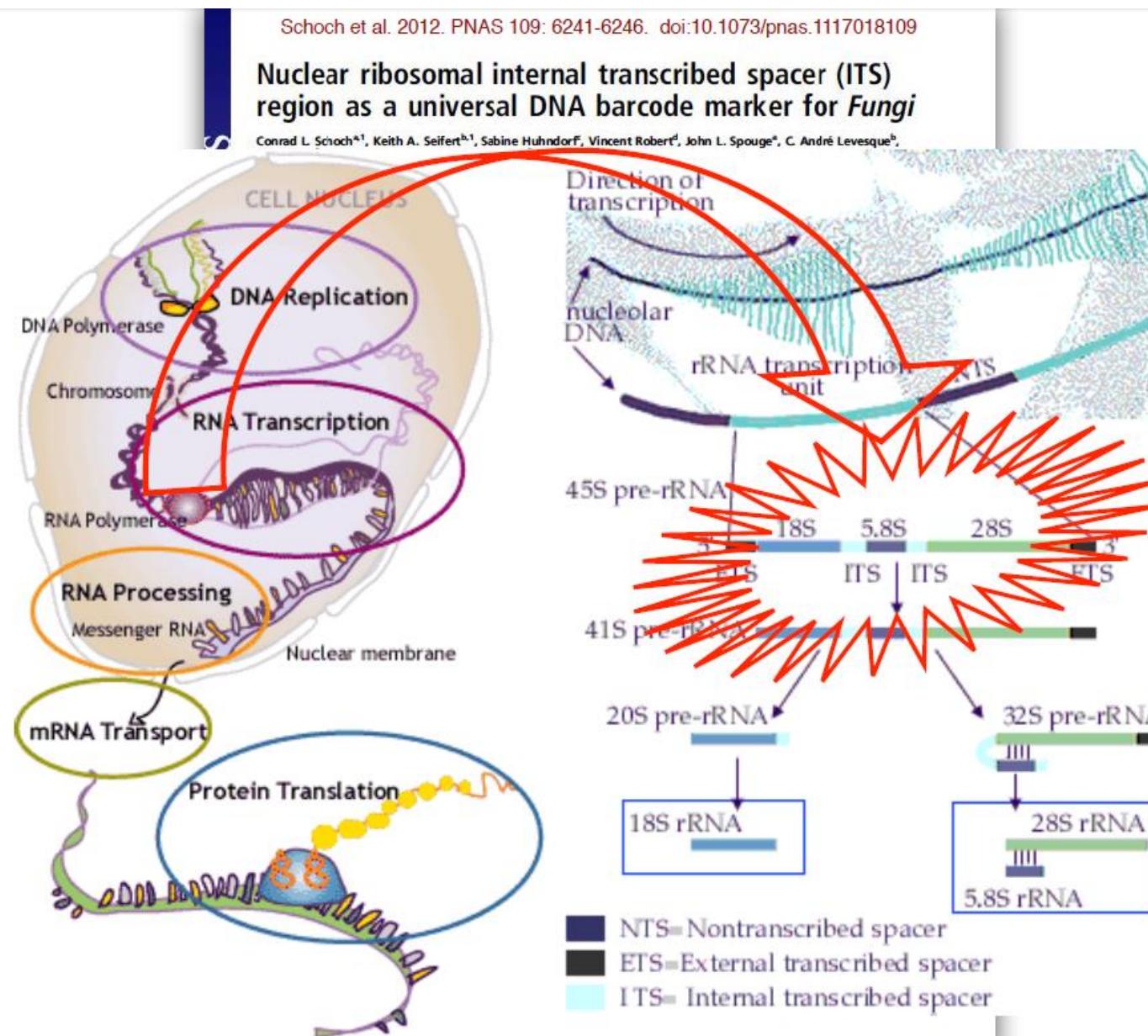
Identification by profiling of secondary metabolites (HPLC)



Sequencing is also valid for moulds



Identification by sequencing – ITS or 28S



Moulds are excellent enzyme producers

- Amylases
 - Transform starch into fermentable carbohydrates
 - Clear starch from fruit syrups
- Cellulases
 - Removal of fibers from e.g. fruit juices
- Pectinases
 - Clarify wines, syrups, vinegar
 - Degrade pectin during coffee fermentation
- Proteases
 - Fish oil from e.g. fish liver

Mould fermented foods

Fermentation pattern:

- solid-state
- liquid-state
- semisolid-state

Use of microorganism(s):

- spontaneous or natural fermentation
- controlled fermentation using starter culture

Substrates:

- | | | |
|------------------|--|---------------|
| • vegetables | • dairy products | • root/tubers |
| • meats | • fish | • others |
| • cereals | • soybean and non-soybean legumes | |

8 min reflection on moulds in fermented food

Form groups of approx. three persons and discuss the following:

- *Which type of mould fermented food or beverages do you know of?*
- *For each of this products what are the positive characteristics?*
- *Do you have any ideas for new innovative food or beverages that could be produced by moulds?*

Prepare yourselves for an open discussion in plenum!

Mould fermented foods – examples of European products



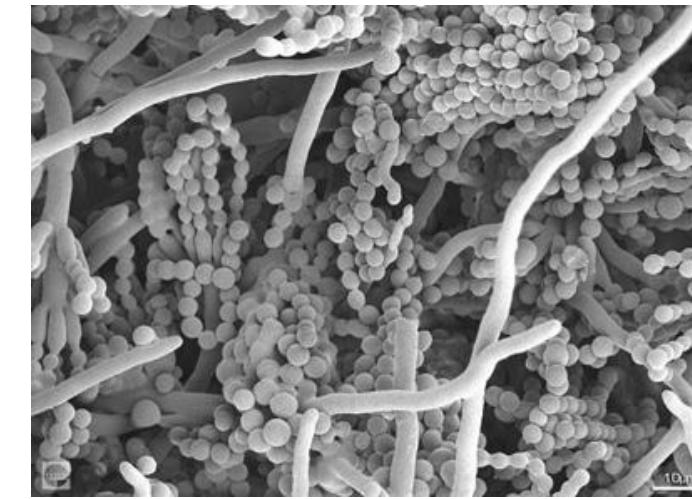
Mould-ripened cheeses

Types

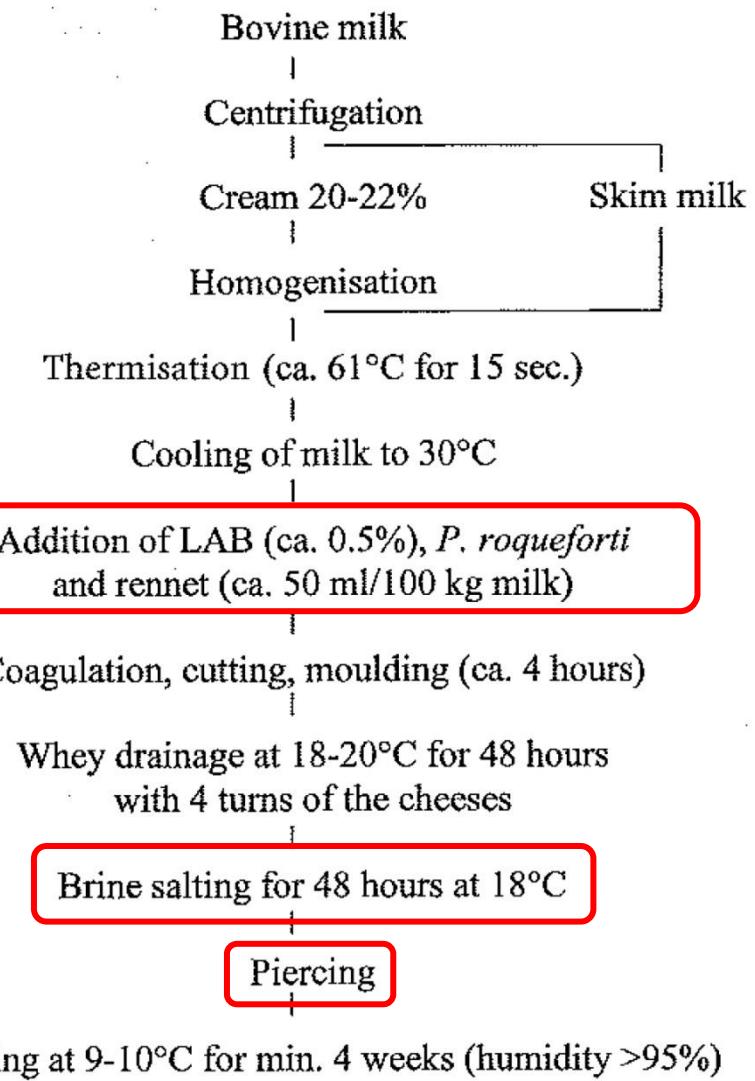
- Mould surface-ripened soft cheese
Ex: Brie, Camembert, goats' milk cheeses (Denmark, France...) a.o.
- Blue-veined cheeses
Ex: Bavarian blue (Germany), Bleu d'Auvergne, Blue des Causses and Roquefort (France), Cabrales (Spain), Gorgonzola (Italy), Danablu (Denmark), Stilton (UK) a.o.

Useful properties – selection of moulds

- Appearance of moulds on/in cheeses (colour of conidia)
- De-acidification activity
- Proteolytic activity
- Lipolytic activity
- Production of aroma
- Interactions with other microorganisms
- Lack of production of mycotoxins



Production of Danablu



Development of *P. roqueforti*

Growth can be divided into three phases: germination, mycelial growth and sporulation

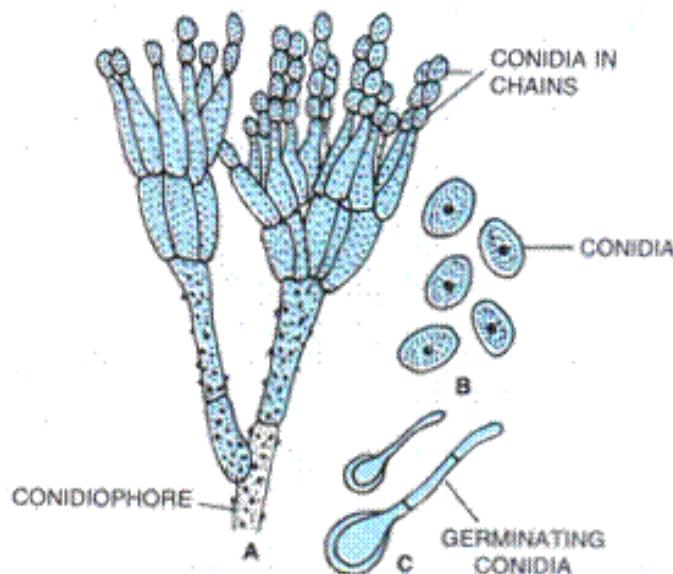
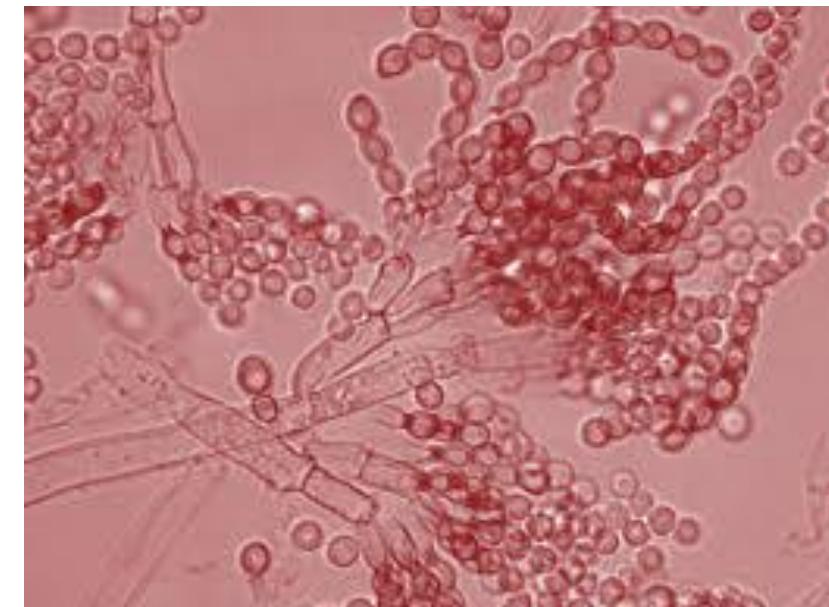


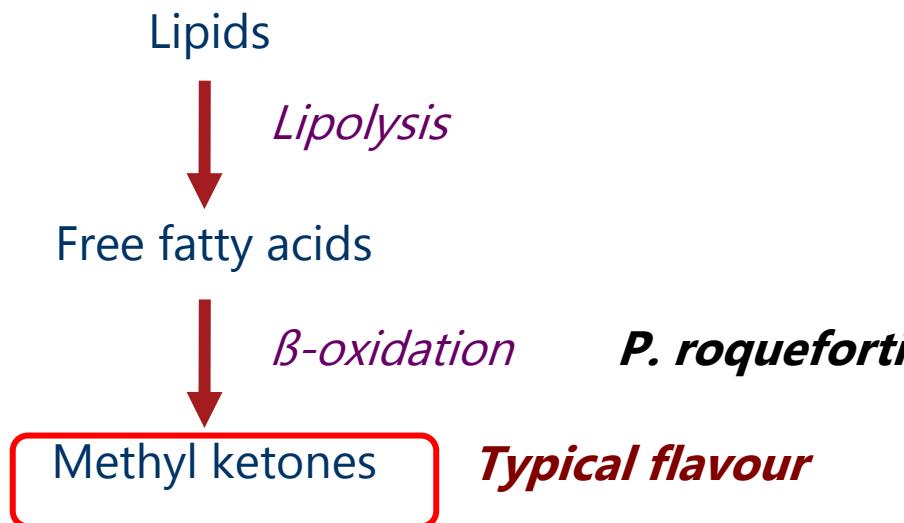
Fig. 1.13. *Penicillium* : Asexual reproduction
A. Conidiophore; B. Conidia; C. Germinating conidi



Lipolysis in Danablu

Lipolytic agents

- Milk lipoprotein lipase
- *P. roqueforti*
- Yeasts
- Lactic acid bacteria



Fat in cheese has influence on:

- Texture
- Flavour



Remarks – moulds in cheese

- *Penicillium roqueforti* is unique for the production of blue mould cheeses whereas *Penicillium camemberti* is unique for production of white mould cheeses
- The growth of moulds can be divided into germination, mycelial growth and sporulation, all important for optimal enzyme production, flavour and appearance
- Strains variations in technological properties occur
- The CO₂/O₂ concentration, pH, temperature and NaCl concentration determine the growth rate of the moulds
- Microbial interactions are important for correct maturation of blue and white mould cheeses

Fermented meat

Mould genera:

- *Penicillium* spp. (*P. camemberti* (*P. candidum*), *P. nalgiovense* and *P. chrysogenum*)
- *Aspergillus* spp.
- *Mucor* spp.
- *Eurotium* spp.
- *Cladosporium* spp.

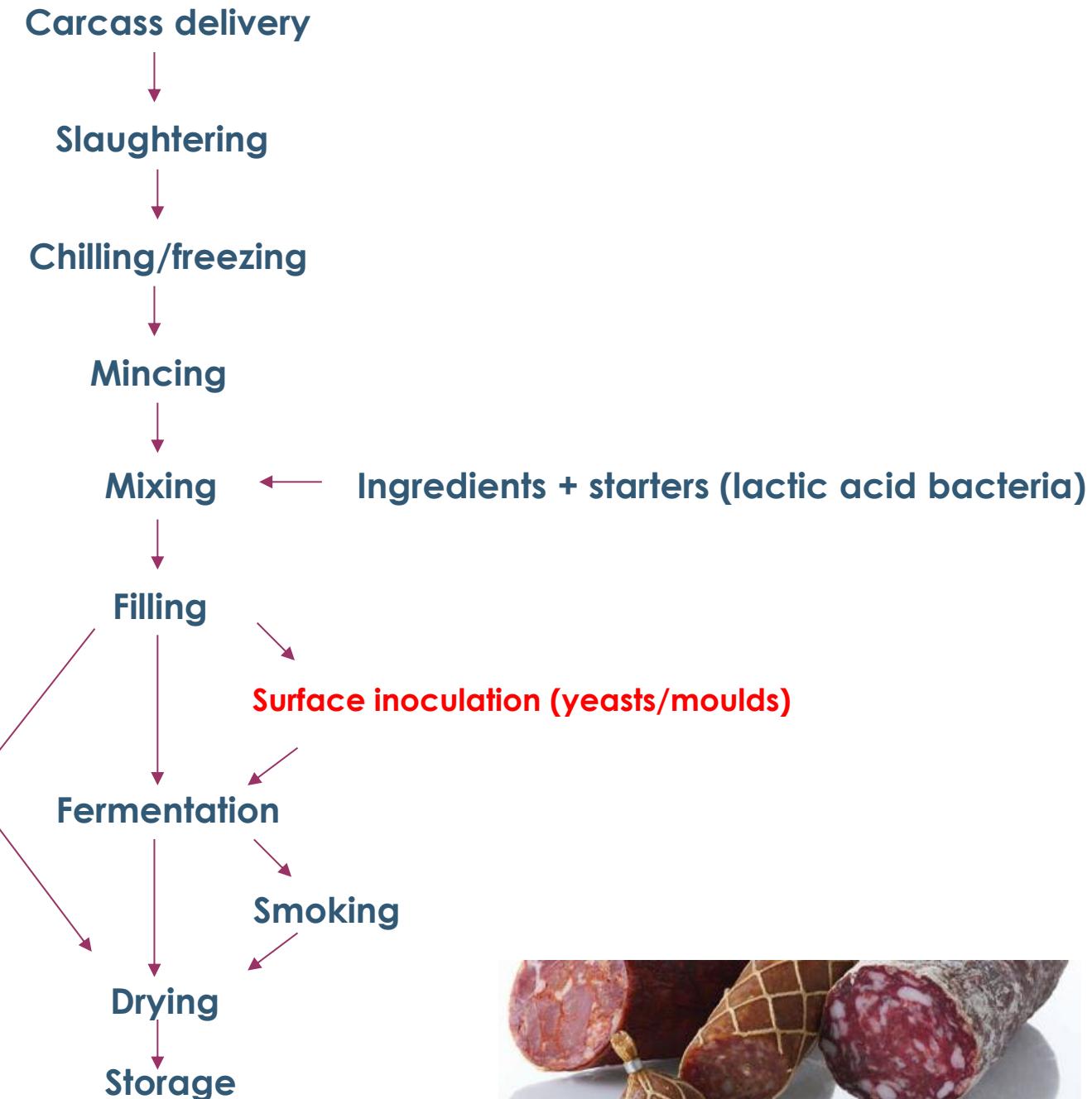
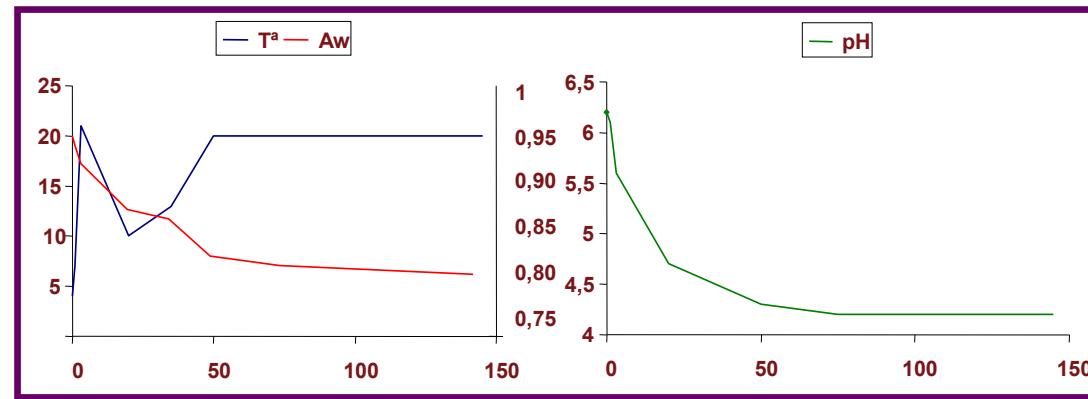


Spanish salchichon

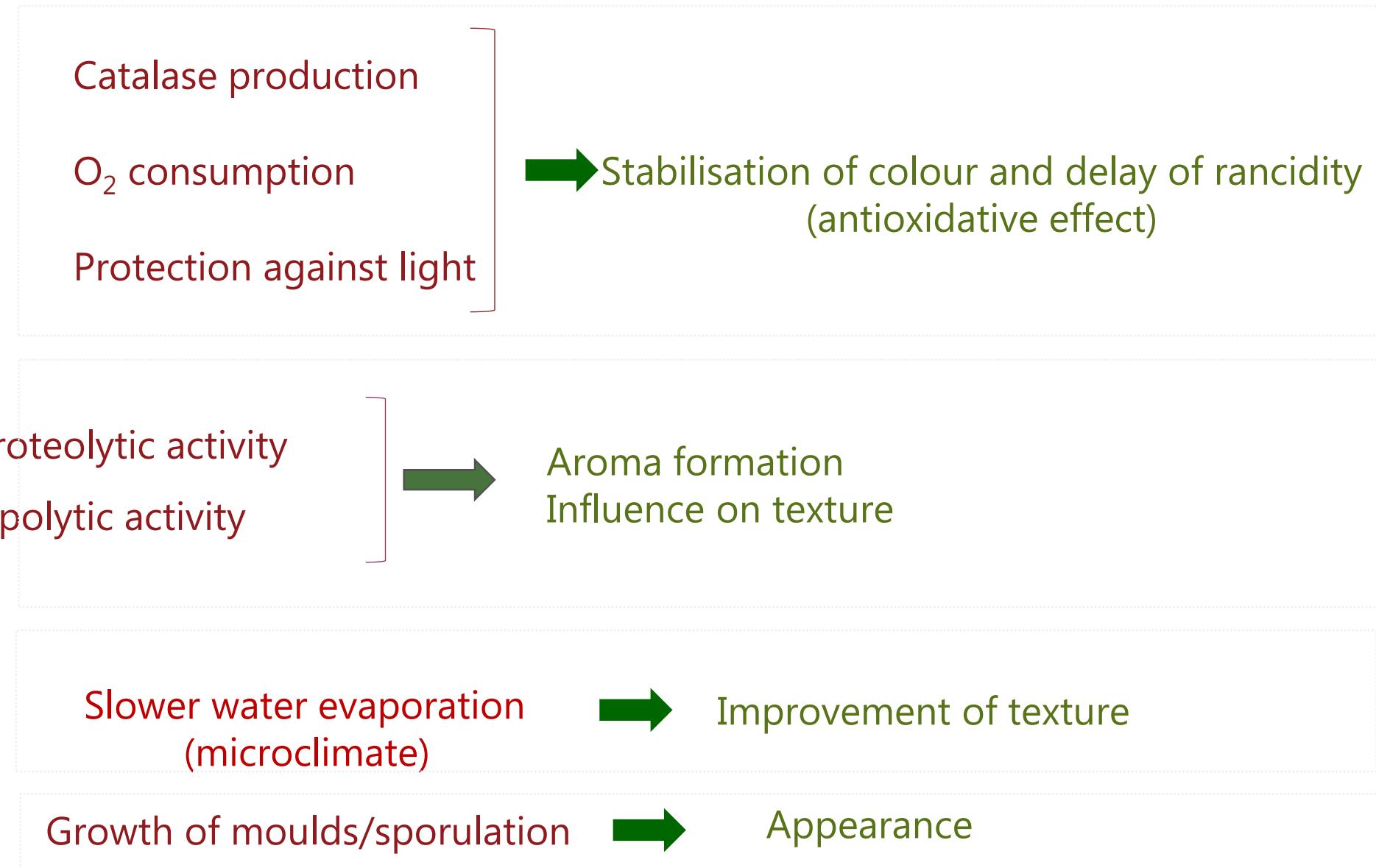
Main roles in general

- Production of extracellular enzymes (amylases, proteases and lipases)
- Improvement of flavour (aroma and taste)
- Antibacterial activity

Flow diagram of fermented sausage



Main role of moulds in fermented sausages

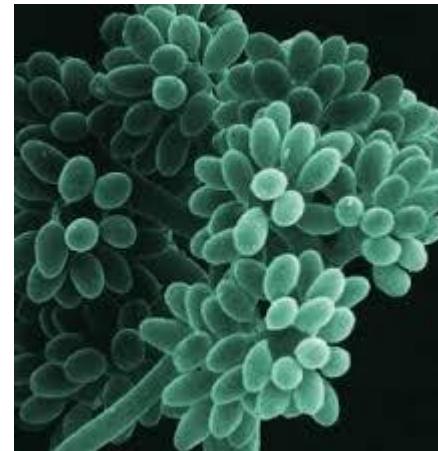


Noble rot – *Botrytis* spp.

- The grey fungus *Botrytis cinerea* cause noble root on wine grapes (may be sprayed on the grapes)
- The mould perforates the grapes' skin, allowing water in the grape to evaporate during dry conditions, and thereby raising the sugar concentration in the remaining juice
- Internationally renowned botrytised wines include the aszú of Tokaj-Hegyalja in Hungary and Slovakia (commonly called Tokaji, Tokajské or Tokay), Sauternes from France and several others



Noble rot on Riesling grapes



Quorn – eat moulds instead of meat!

- Quorn mycoprotein (*Fusarium venenatum*)

The mould is grown in continually oxygenated water in large fermentation tanks. Glucose and fixed nitrogen are added, as are vitamins and minerals. The resulting mycoprotein is then extracted and heat treated.

The mycoprotein is dried and mixed with e.g. egg albumen, which acts as a binder. It is then textured, giving it some of the grained character of meat, and pressed either into a mince resembling ground beef, chicken breasts, meatballs or e.g. turkey roasts.

Adverse effects e.g. allergens have been reported.



Quorn fillets



Quorn Vegan Burger

Mould fermented foods – examples of Asian and Latin American products



Koji – an inoculum

- Saccharifyer of starch to glucose in Japanese fermented foods (like malt in Western fermented foods). Kouji is a similar inoculum product in China.
- Koji is made by proliferating traditional filamentous *Aspergillus oryzae* on steamed rice.
- Following saccharification yeasts (mostly *S. cerevisiae*) are often added to complete the fermentation (depends on the products).
- Koji is used for a variety of products e.g. sake, miso, soy sauce, vinegar etc.



Miso

Soybeans (or rice and barley) fermented by adding salt and
Aspergillus oryzae or *Aspergillus sojae*



Japan

Miso is made from "koji" which is prepared from steamed rice (barley or soybean) and wheat inoculated with koji fungi. Maillard reaction occurs during 5-11 months of ripening.



Akamiso, "red miso"
Maillard reaction

Miso is used in especially soups and broths

Similar products:



Soybean Jang (China)



Doenjang (South Korea)



Taosi (Philippines)

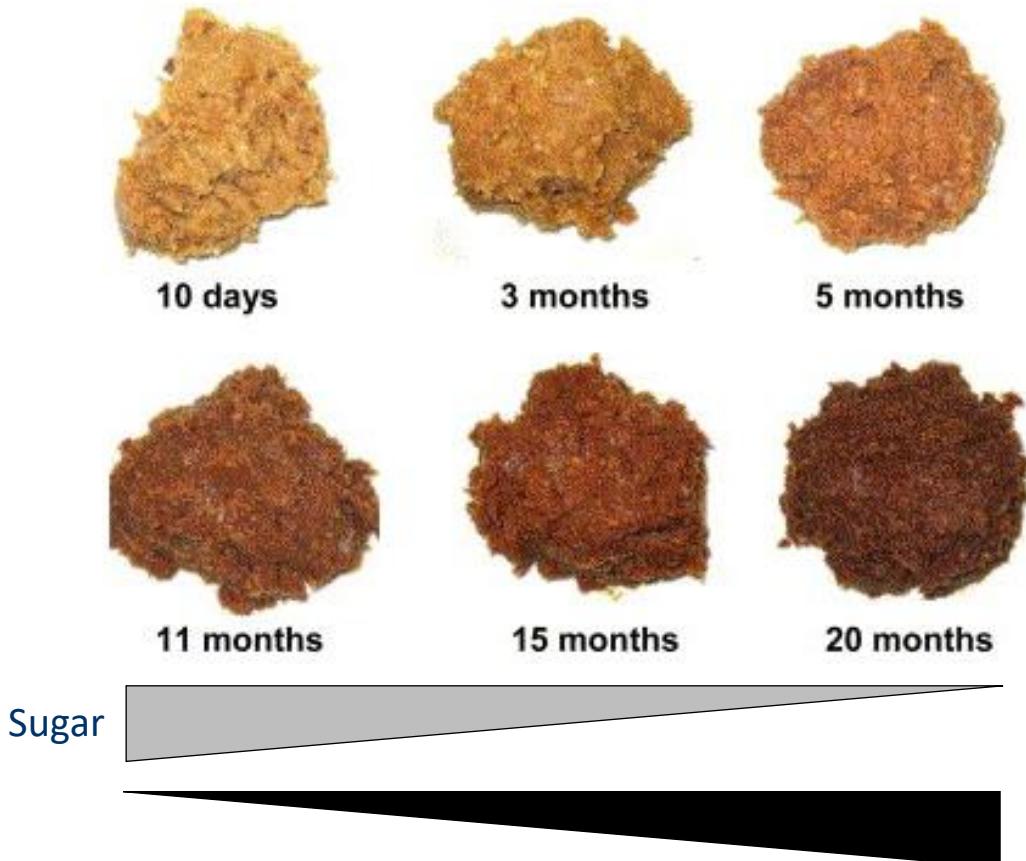


Taoco (Indonesia)



Shiromiso, "white miso"

Miso quality depends on time of maturation



Available online at www.sciencedirect.com



Food Chemistry 99 (2006) 736–741

Food
Chemistry
www.elsevier.com/locate/foodchem

Taste enhancer from the long-term ripening of miso (soybean paste)

Masashi Ogasawara *, Yuki Yamada, Makoto Egi

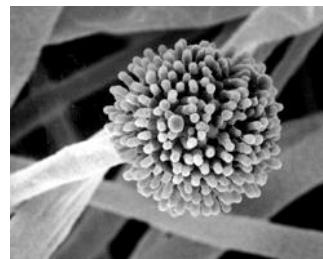
Food Creation Center, Kyowa Hakko Food Specialties Co., Ltd., 4041 Ami, Ami-machi, Inashiki-gun, Ibaraki Prefecture 300-0398, Japan

Received 25 April 2005; received in revised form 26 August 2005; accepted 26 August 2005

Soy sauce

Aspergillus oryzae or *Aspergillus sojae*

A. oryzae has higher α -amylase productivity (works well with yeast/alcoholic fermentation, whereas *A. sojae* exhibits higher endopolygalacturonase productivity (more versatile aroma formation).



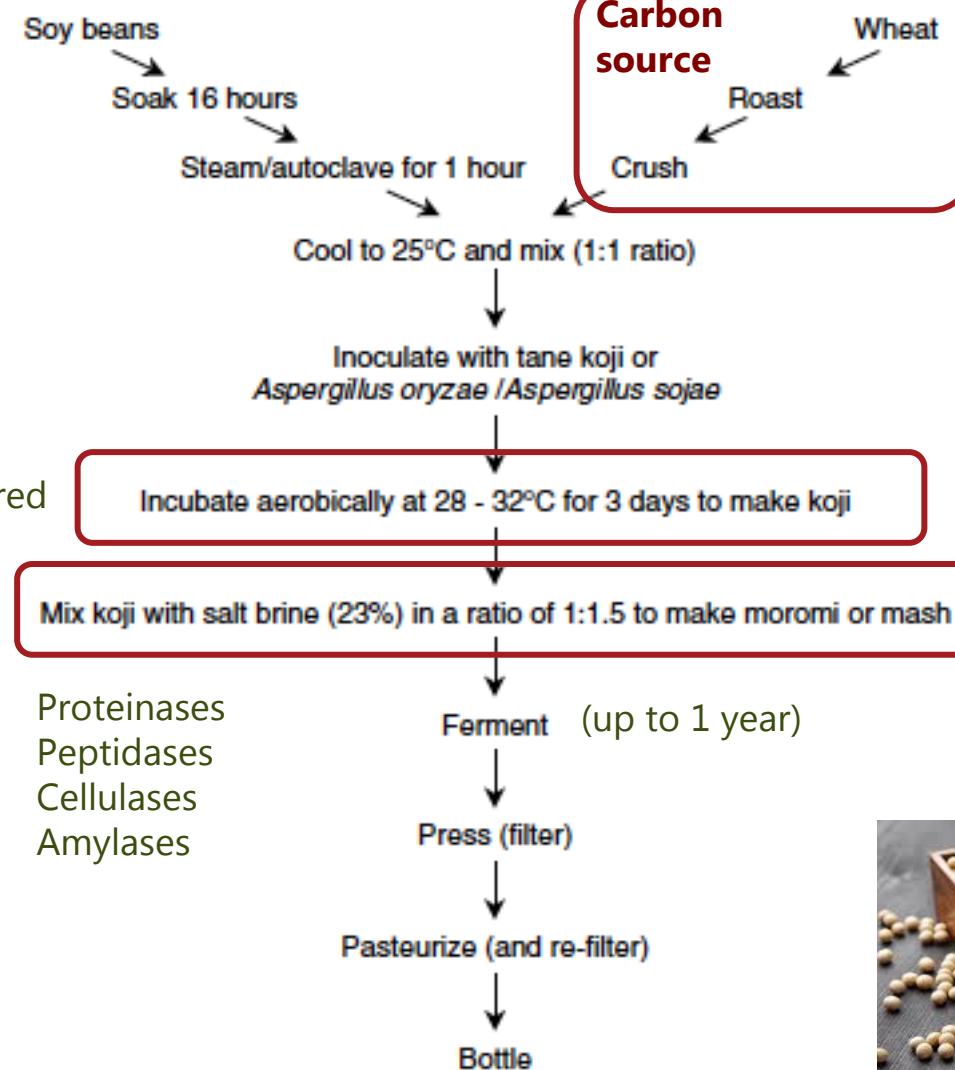
Light or fresh soy sauce (生抽 shēng chōu)

Dark and old soy sauce (老抽 lǎo chōu)



Nitrogen source

Flavour
Colour
Enhances mould growth



Tempeh



Mould-fermented soybean made by *Rhizopus microsporus* var. *oligosporus* (starter cultures)
Spontaneously also: *R. arrhizus*, *R. delemar* etc.

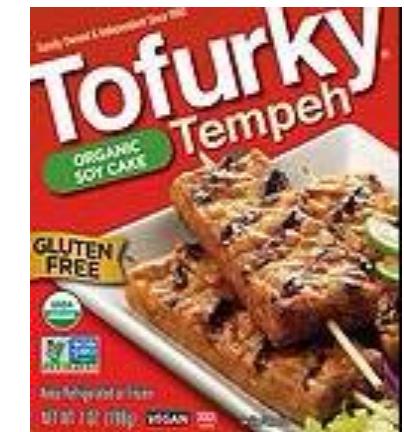
Tempeh is a traditional fermented food made from soaked and cooked soybeans inoculated with moulds, usually of the genus *Rhizopus*. After fermentation has occurred, the soybeans are bound together into a compact cake and wrapped in e.g. banana leaves.



Nordic tempeh



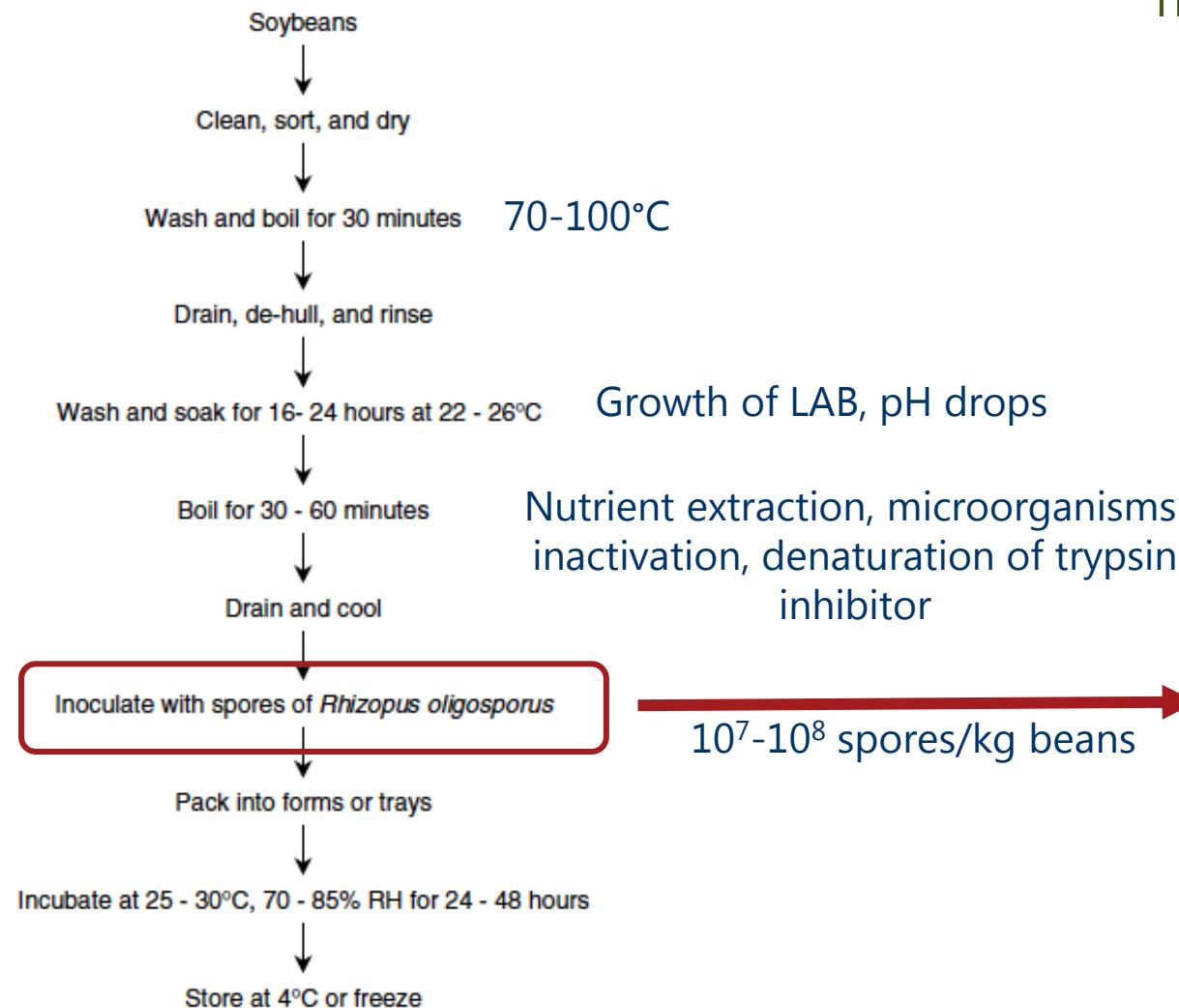
Rich source of protein (19%),
vitamin B₁₂ (important in
vegetarian diets)



Tempeh



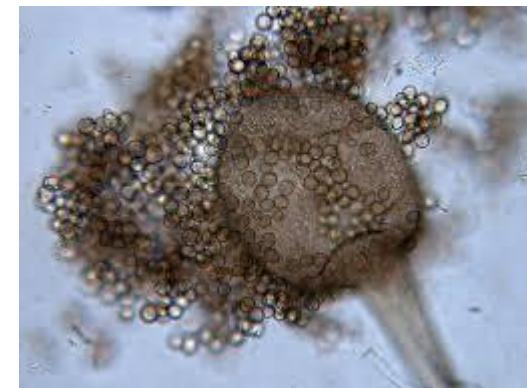
Indonesia



Traditional tempeh is often produced using *Hibiscus tiliaceus* leaves.



Modern fermentation uses spores cultivated in a laboratory



Red "yeast" rice

Dressing mixture:

When boiled white rice is cultivated with *Monascus purpureus* for about 3 weeks, the whole rice grain turns red and become fragile for grinding.

Then brine is mixed with the "red rice" powder and can be used to dress fermented tofu (made from soy beans).



Packed in glass bottles and used as a condiment



Monascus purpureus



Monascus purpureus is a species of mould that is purplish-red in color.



China

Rice cake starter cultures for alcoholic beverages and food

Amylolytic starter for rice wine preparation

Uncooked rice flour mixed
with herbs and spices



Dough (flattened discs)



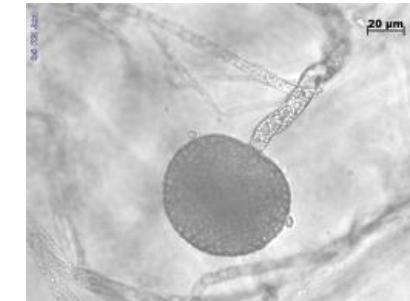
Sprinkled with previously
powdered *ragi* or *men*



Incubation



Dehydration



Inoculation with fungal spores
(*Amylomyces rouxii* and *Rhizopus* spp.)



Indonesia
Vietnam
Cambodia

Tapuy



Philippines

Traditional alcoholic drink popular in the mountains of northern Philippines



Huitlacoche [wee-tlah-KOH-cheh] - Mexico



Basidiomycetes (*Ustilago maydis*) that grows as a parasite on cobs of pre-harvest maize

The large fruiting body is edible, and is locally known as caviar *azteca*, *huitlacoche* or "maize mushroom"

Rich in carbohydrates, proteins, fats, vitamins, and minerals as well as in essential amino acids (especially lysine) and fatty acids (linoleate)



In conclusion…

- ✓ Moulds are used worldwide for production of safe and tasty food and beverages;
- ✓ Moulds produce a variety of enzymes and secondary metabolites compared to bacteria and yeasts;
- ✓ Common moulds used in fermented food and beverages are *Penicillium*, *Aspergillus*, *Rhizopus*, *Mucor* and *Botrytis* spp. *Fusarium venenatum* can be used for production of mycoproteins;
- ✓ Identification of moulds is based on micro- and macromorphology, their production of secondary metabolites as well as sequence analyses of predominantly the ITS regions of the rRNA gene;
- ✓ Several moulds are mycotoxin producers so careful identification should be ensured before they are used as starter cultures for food production. The ability to produce mycotoxins might change depending on the composition of the food matrix.

From 1st to 3rd Generation DNA Sequencing strategies

Lukasz Krych Associate Professor (krych@food.ku.dk)

Fermented Foods and Beverages 2024

UNIVERSITY OF COPENHAGEN

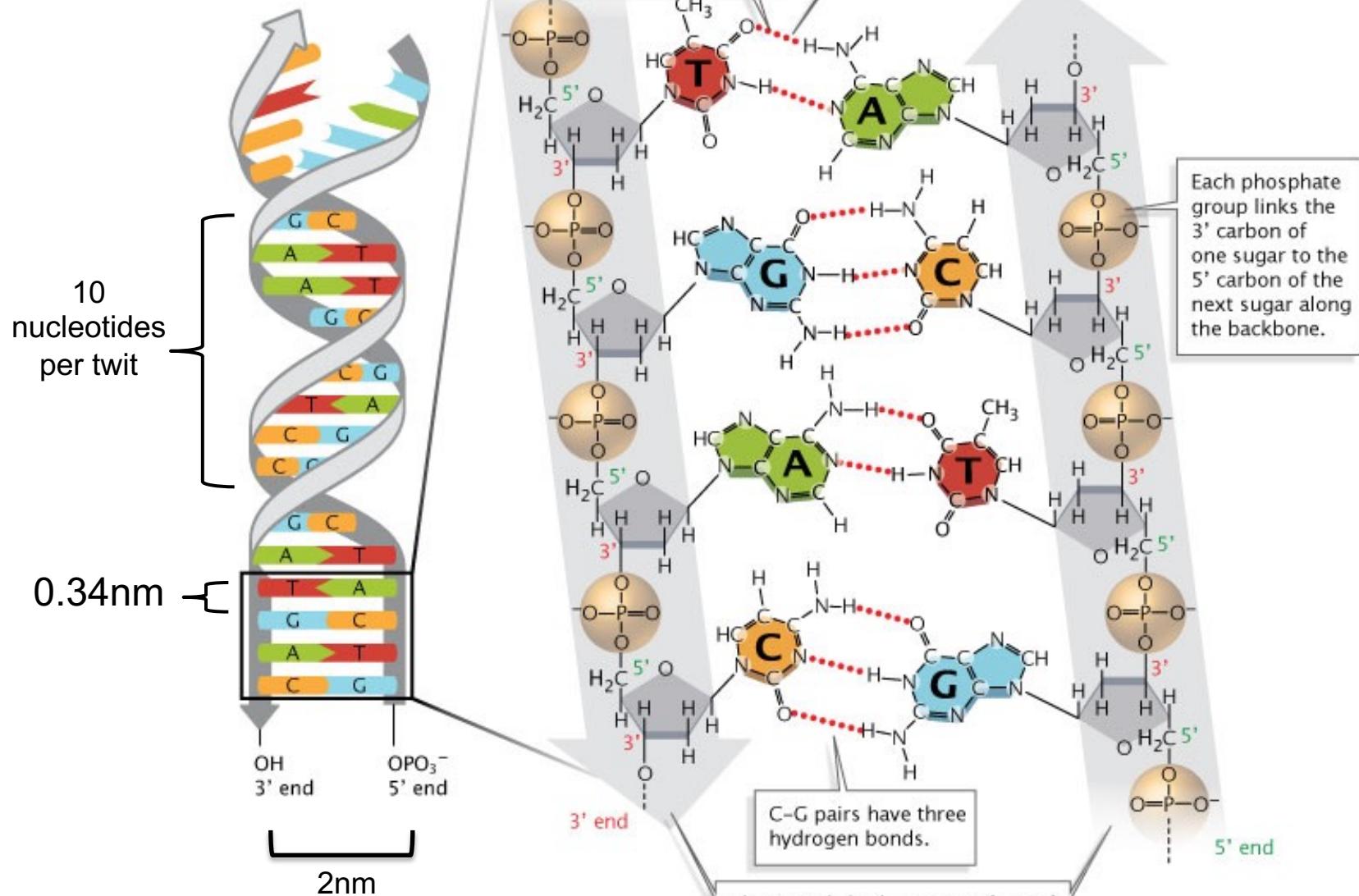


SIC
SIS

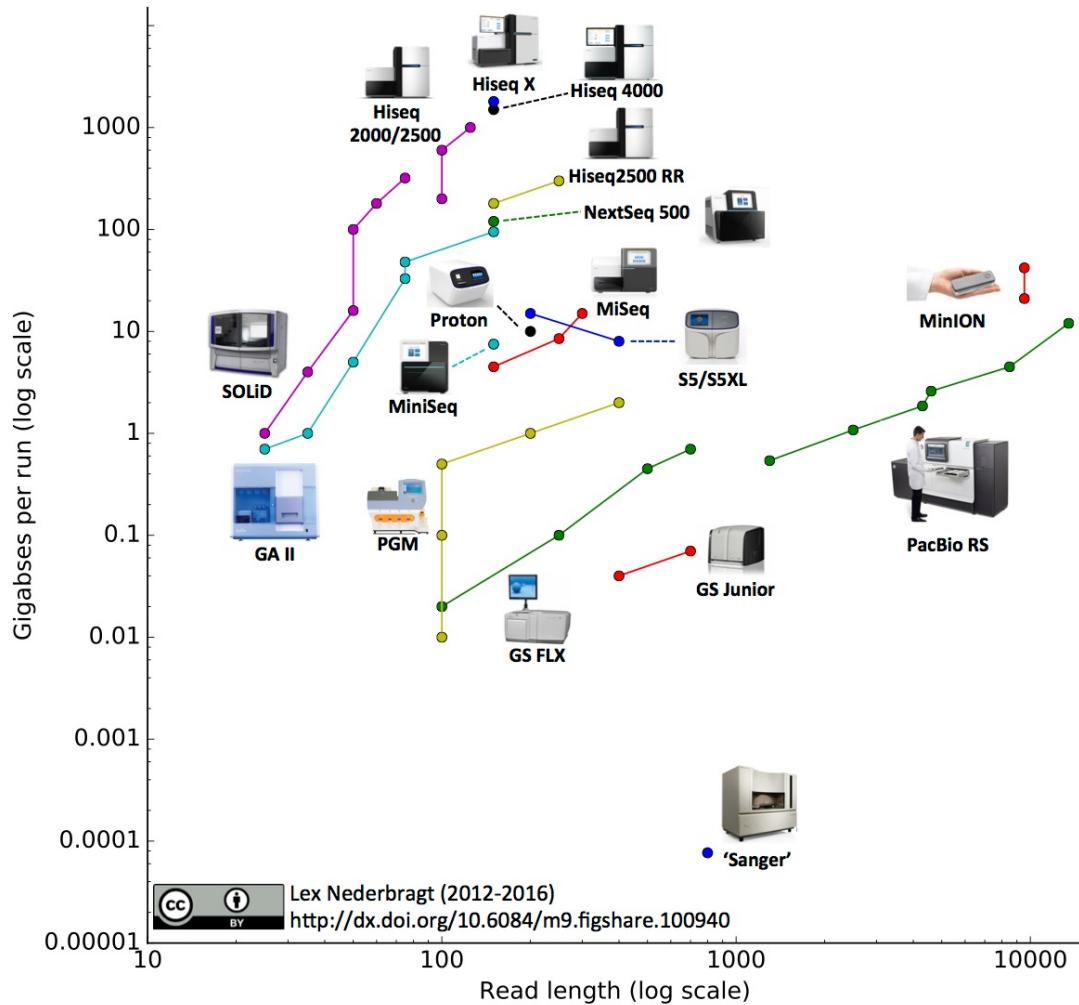


B
C
G

Right-handed helix of DNA

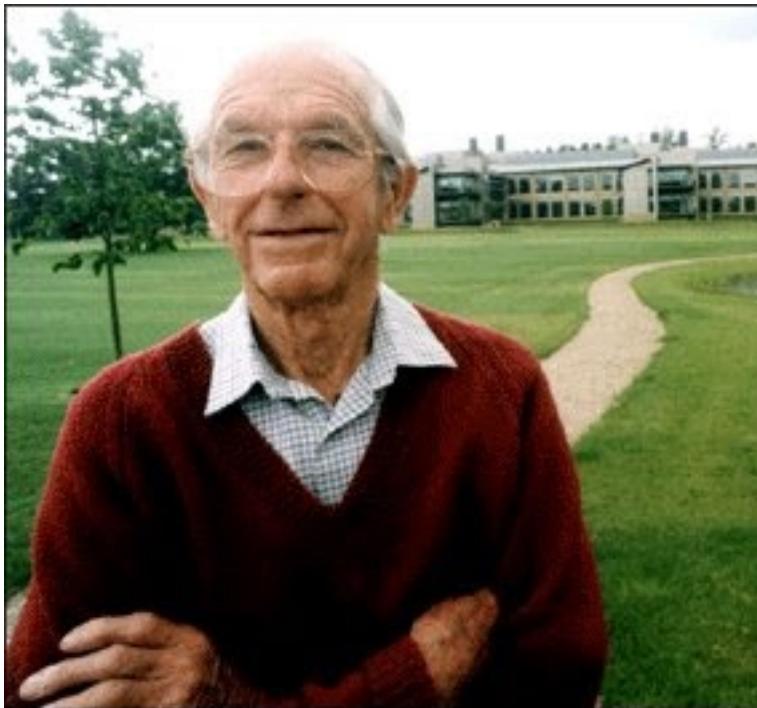


Next generation Sequencing



Sanger Sequencing of DNA

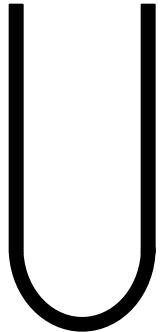
1st generation sequencing



*Frederick Sanger 1918-
2013*

TACAACTGAGCGACT
ATGTTGACTCGCTGA

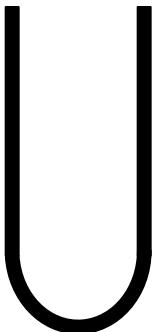
-A



ATGTTGACTCGCTG

ATGTTG

-T



ATGTTGACTCGC

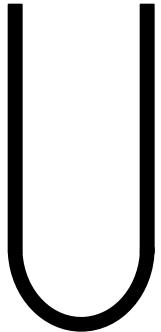
ATGTTGAC

ATGT

ATG

A

-C

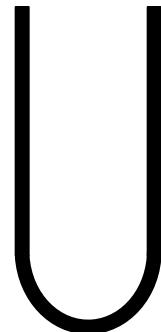


ATGTTGACTCG

ATGTTGACT

ATGTTGA

-G



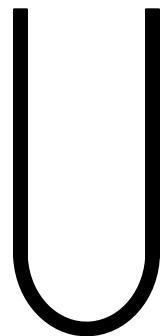
ATGTTGACTCGCT

ATGTTGACTC

ATGTT

AT

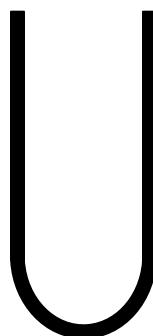
-A



ATGTTGACTCGCTG

ATGTTG

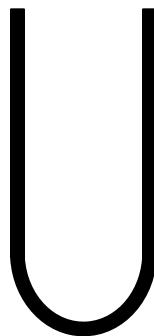
-T



ATGTTGACTCGC

ATGTTGAC

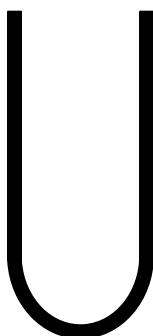
-C



ATGTTGACTCG

ATGTTGACT

-G



ATGTTGACTCGCT

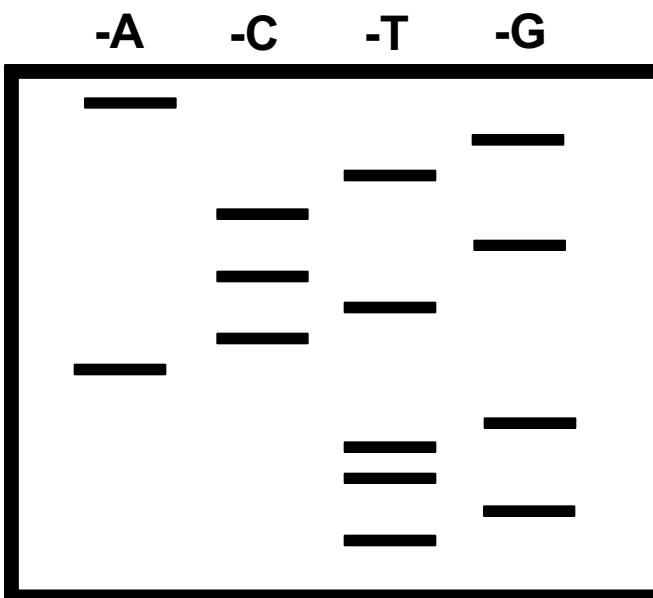
ATGTTGACTC

ATGT

ATG

A

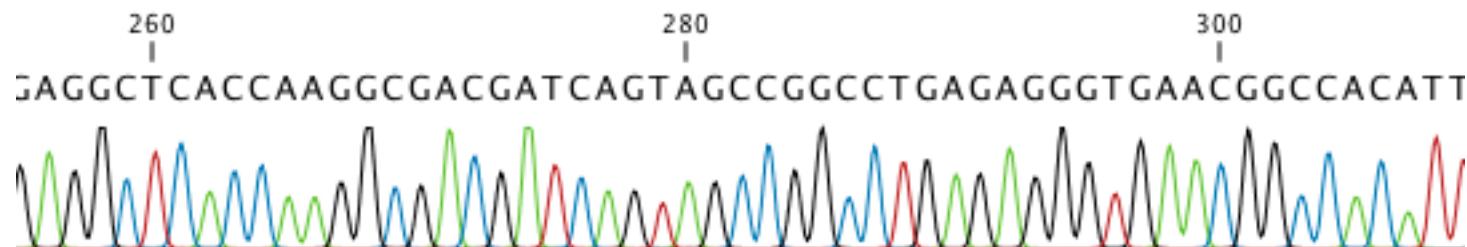
TGTTGACTCGCTGA



1986



ddATP*
ddCTP*
ddTTP*
ddGTP*



- 3.3 billion base-pairs
- With 100x coverage it would take > 30 years to sequence one genome with highest throughput platform



Technology speeds science. ABI sequencers at Venter Institute, 2007.

Next Generation Sequencing



- 1990-2003
6 countries x 13 years = 1 human genome
Cost ~ 3 billion \$



- 2005
1 platform x few weeks = 1 genome
cost ~ < \$1,000,000





- 2022
HiSeqX = ~50 genomes/day
cost < \$1000 /genom

650 years of international effort with technology from 90s in 1 day

3rd Generation Sequencing

Guinness World Record Awarded for Fastest DNA Sequencing — Just 5 Hours

Record was set by researchers from Stanford University, with collaborators from NVIDIA, Oxford Nanopore Technologies, Google, Baylor College of Medicine and the University of California at Santa Cruz.

February 18, 2022 by ISHA SALIAN





MiSeq
Focused power. Speed and simplicity for targeted and small genome sequencing.



NextSeq 500
Flexible power. Speed and simplicity for everyday genomics.



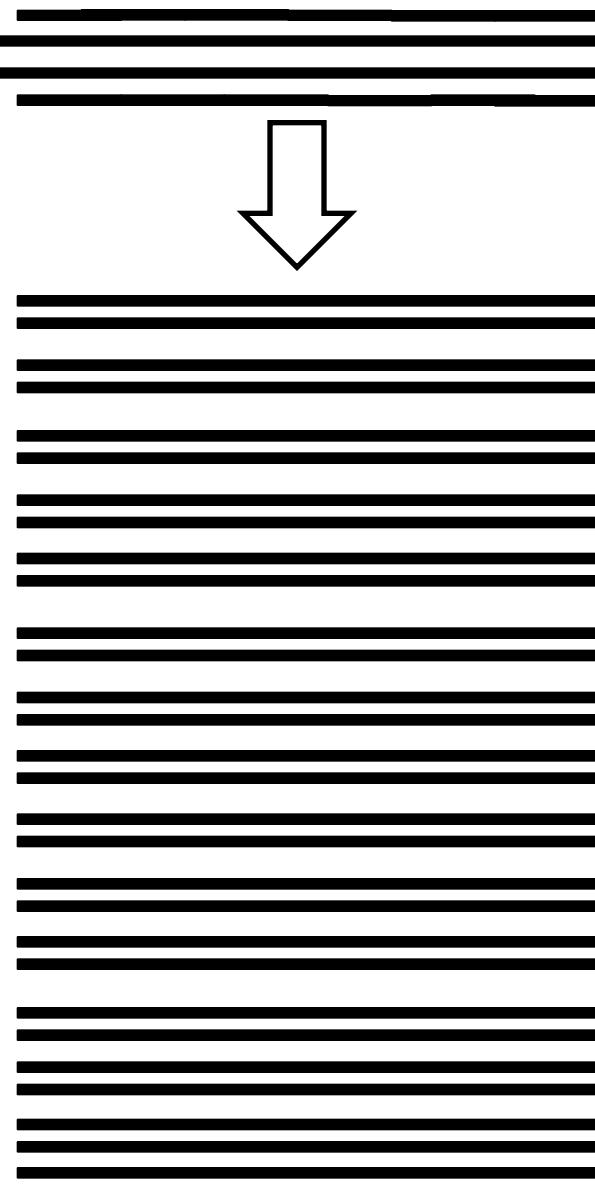
HiSeq 2500
Production power. Power and efficiency for large-scale genomics.



HiSeq X*
Population power. \$1,000 human genome and extreme throughput for population-scale sequencing.

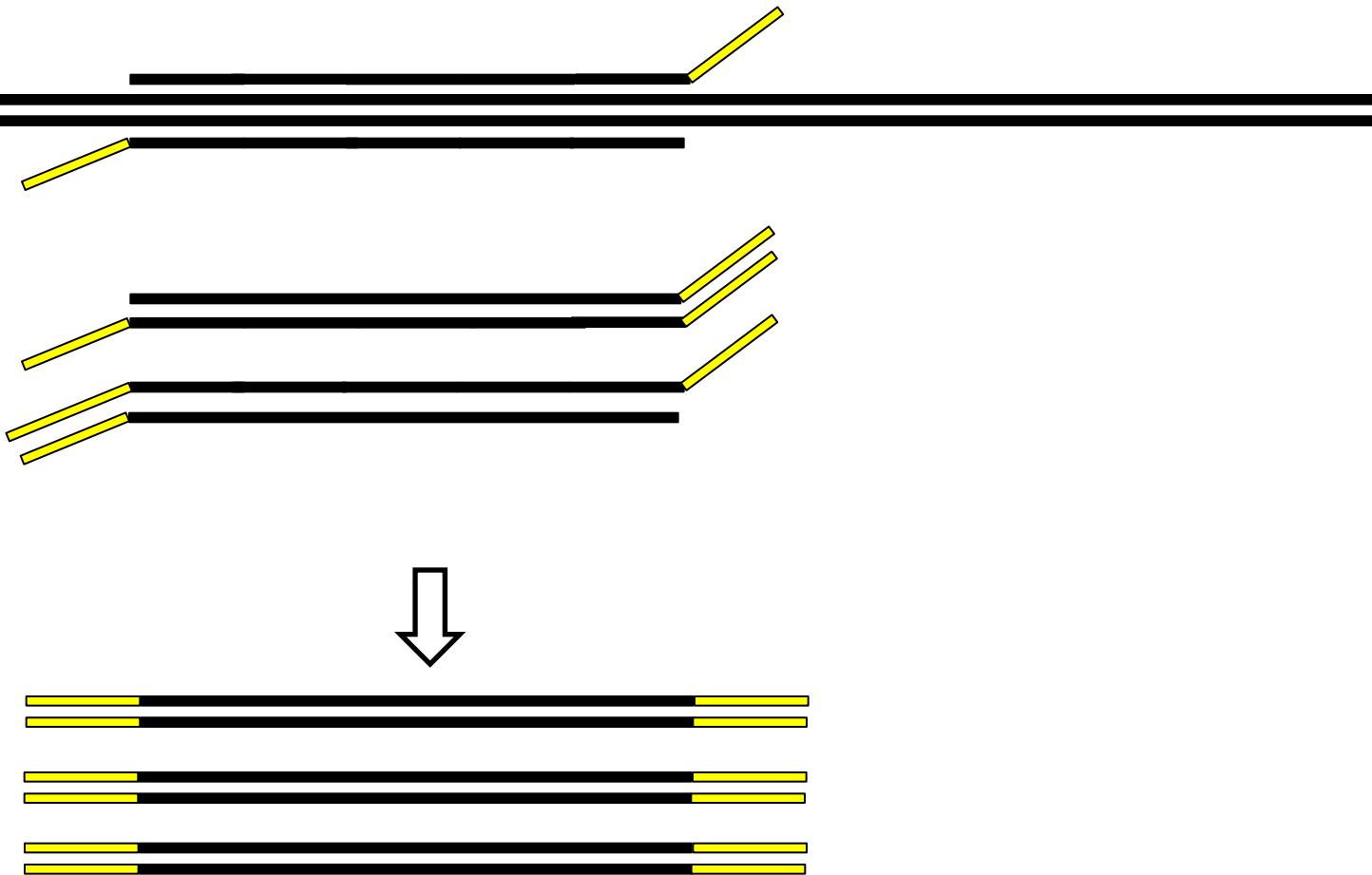
Key applications	Small genome, amplicon, and targeted gene panel sequencing.	Everyday genome, exome, transcriptome sequencing, and more.	Production-scale genome, exome, transcriptome sequencing, and more.	Population-scale human whole-genome sequencing.
Run mode	N/A	Mid-Output	High-Output	Rapid Run
Flow cells processed per run	1	1	1	1 or 2
Output range	0.3-15 Gb	20-39 Gb	30-120 Gb	10-180 Gb
Run time	5-55 hours	15-26 hours	12-30 hours	7-40 hours
Reads per flow cell†	25 Million‡	130 Million	400 Million	300 Million
				2 Billion
Maximum read length	2 × 300 bp	2 × 150 bp	2 × 150 bp	2 × 125 bp
				2 × 150 bp

PCR



Library preparation

PCR1



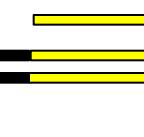
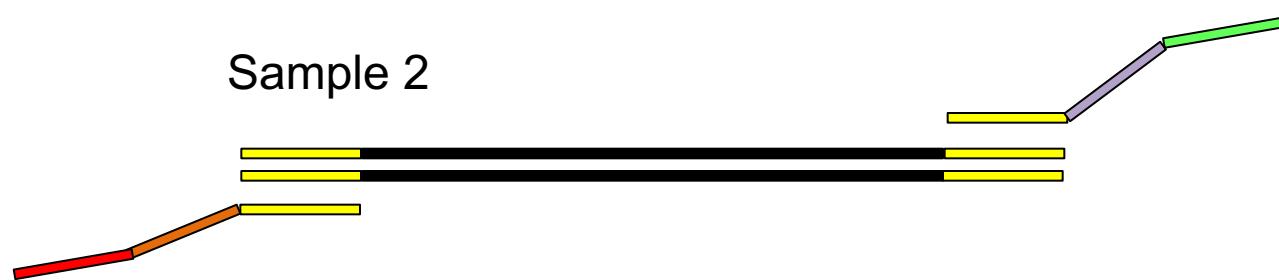
Library preparation

PCR2

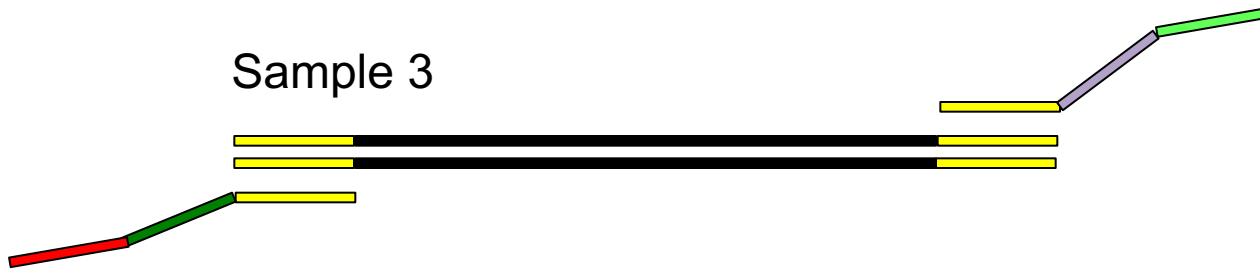
Sample 1



Sample 2

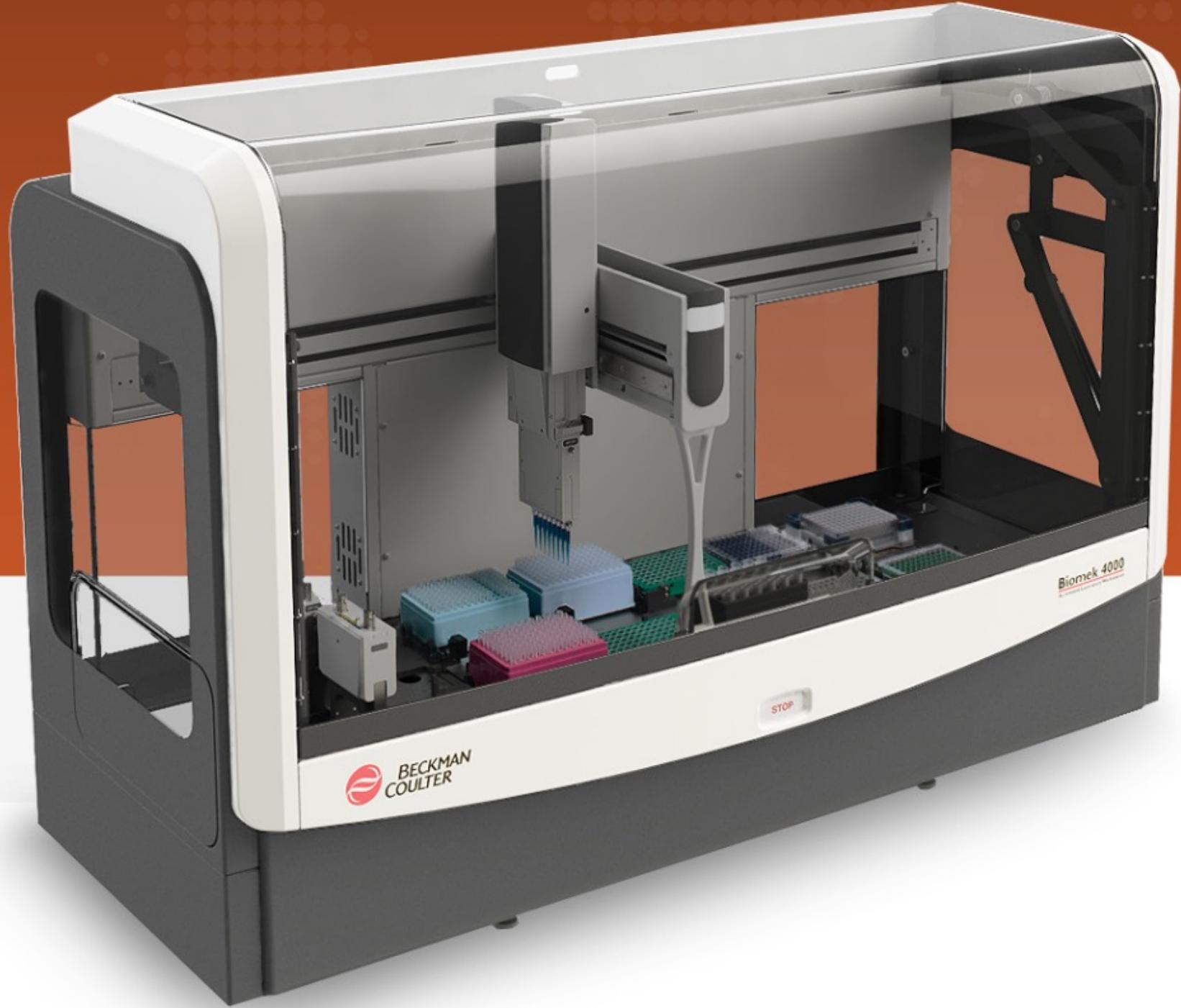


Sample 3

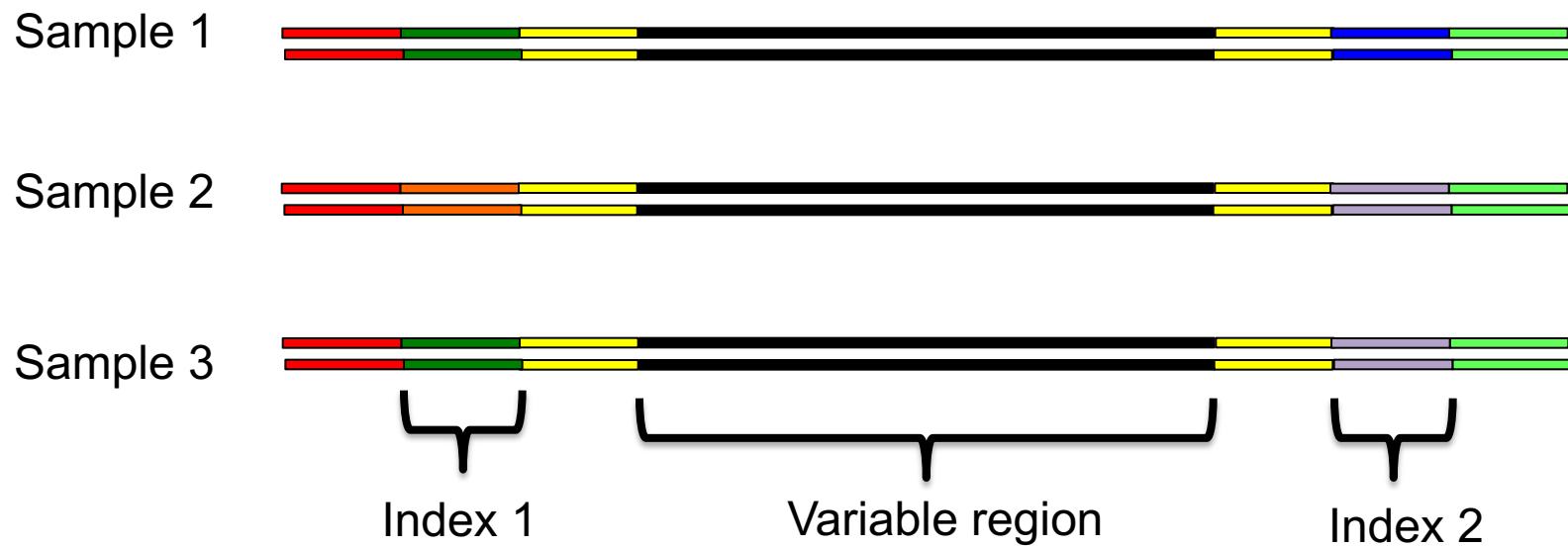


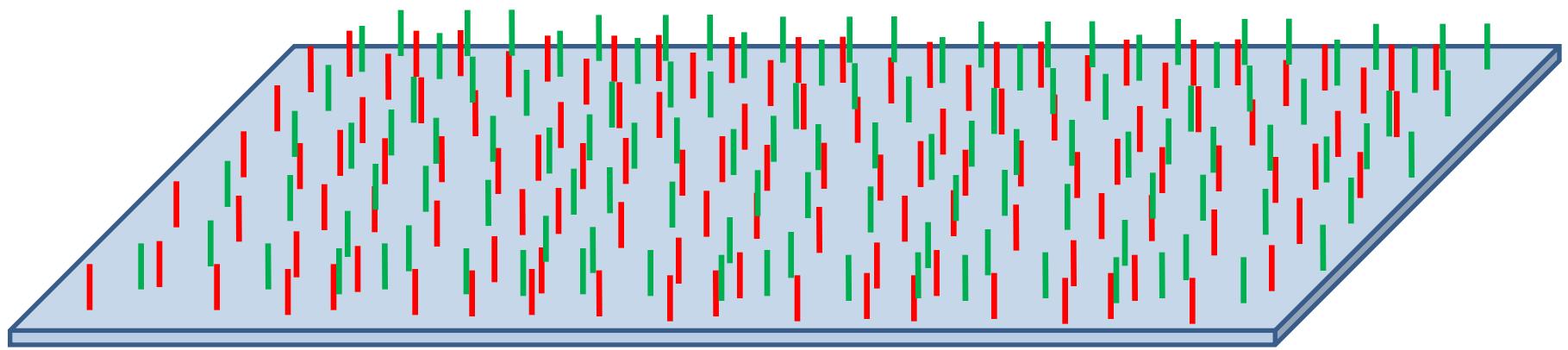
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S501	1	2	3	4	5	6	7	8	9	10	11	12	193	194	195	196	197	198	199	200	201	202	203	204
S502	13	14	15	16	17	18	19	20	21	22	23	24	205	206	207	208	209	210	211	212	213	214	215	216
S503	25	26	27	28	29	30	31	32	33	34	35	36	217	218	219	220	221	222	223	224	225	226	227	228
S504	37	38	39	40	41	42	43	44	45	46	47	48	229	230	231	232	233	234	235	236	237	238	239	240
S505	49	50	51	52	53	54	55	56	57	58	59	60	241	242	243	244	245	246	247	248	249	250	251	252
S506	61	62	63	64	65	66	67	68	69	70	71	72	253	254	255	256	257	258	259	260	261	262	263	264
S507	73	74	75	76	77	78	79	80	81	82	83	84	265	266	267	268	269	270	271	272	273	274	275	276
S508	85	86	87	88	89	90	91	92	93	94	95	96	277	278	279	280	281	282	283	284	285	286	287	288
S510	97	98	99	100	101	102	103	104	105	106	107	108	289	290	291	292	293	294	295	296	297	298	299	300
s511	109	110	111	112	113	114	115	116	117	118	119	120	301	302	303	304	305	306	307	308	309	310	311	312
s513	121	122	123	124	125	126	127	128	129	130	131	132	313	314	315	316	317	318	319	320	321	322	323	324
s515	133	134	135	136	137	138	139	140	141	142	143	144	325	326	327	328	329	330	331	332	333	334	335	336
s516	145	146	147	148	149	150	151	152	153	154	155	156	337	338	339	340	341	342	343	344	345	346	347	348
s517	157	158	159	160	161	162	163	164	165	166	167	168	349	350	351	352	353	354	355	356	357	358	359	360
s518	169	170	171	172	173	174	175	176	177	178	179	180	361	362	363	364	365	366	367	368	369	370	371	372
s520	181	182	183	184	185	186	187	188	189	190	191	192	373	374	375	376	377	378	379	380	381	382	383	384
s521	385	386	387	388	389	390	391	392	393	394	395	396	409	410	411	412	413	414	415	416	417	418	419	420
s522	397	398	399	400	401	402	403	404	405	406	407	408	421	422	423	424	425	426	427	428	429	430	431	432

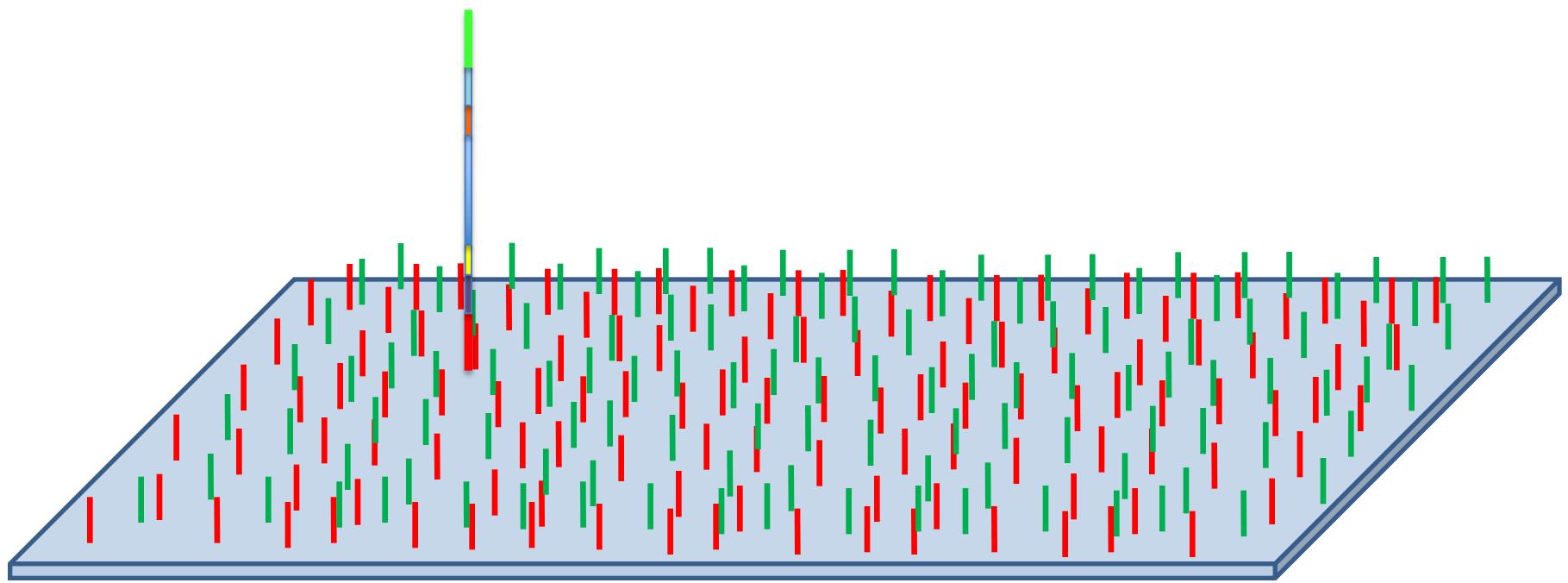
Up to 432 samples

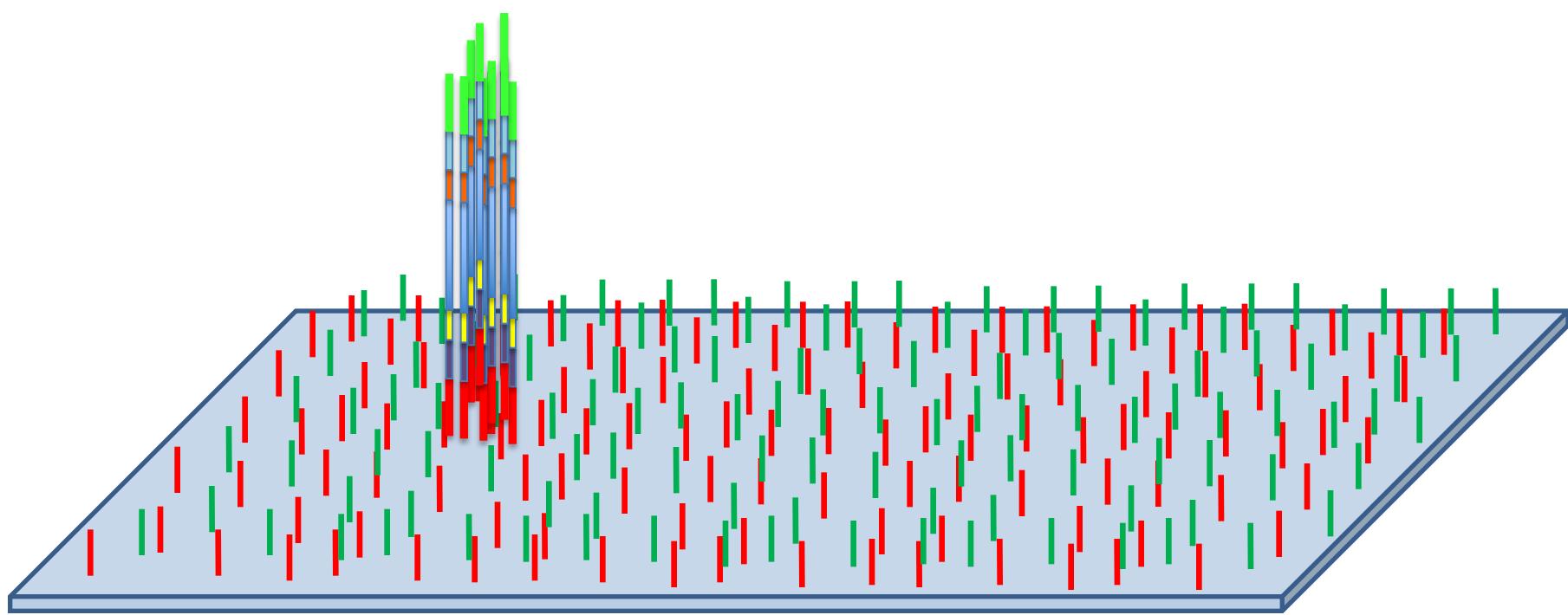


Library

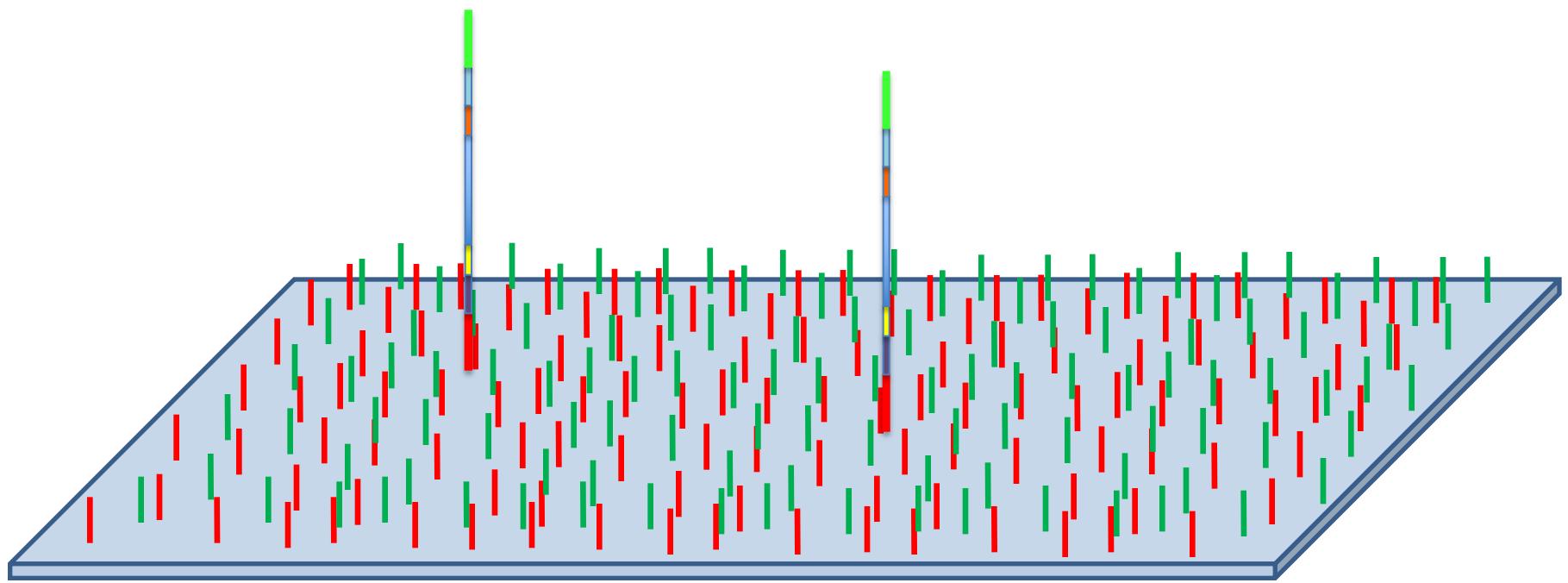


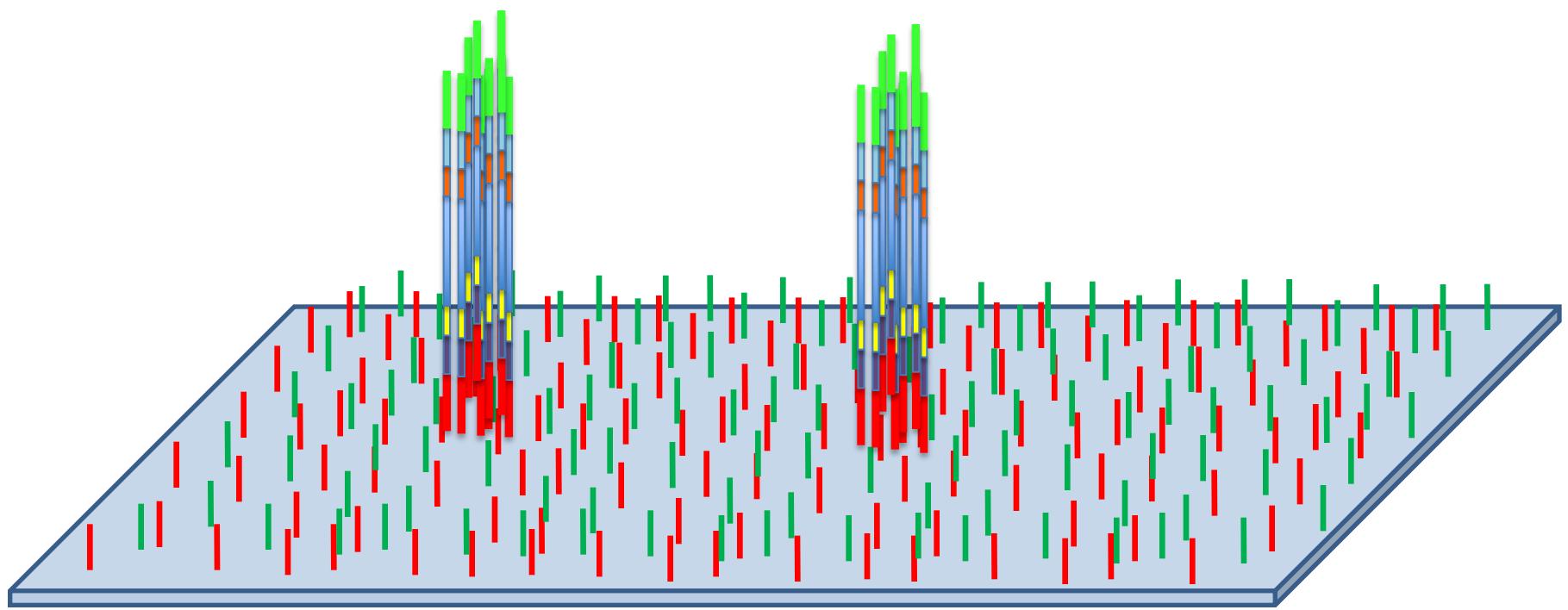


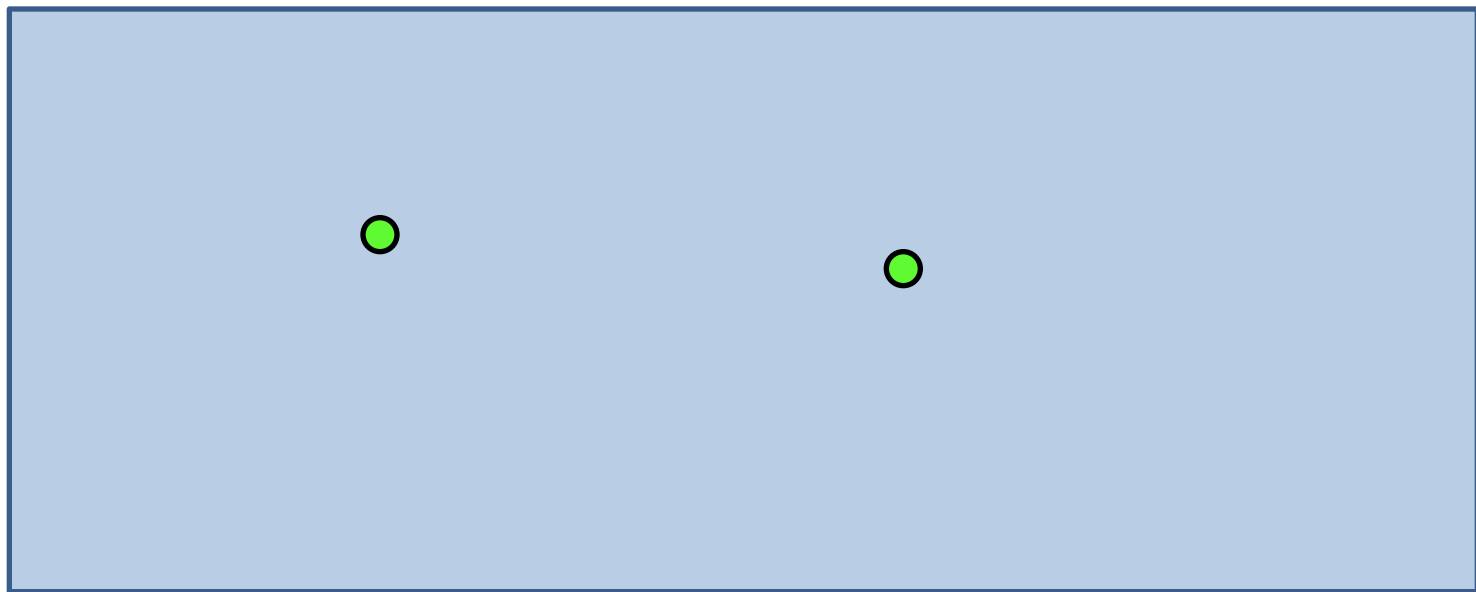


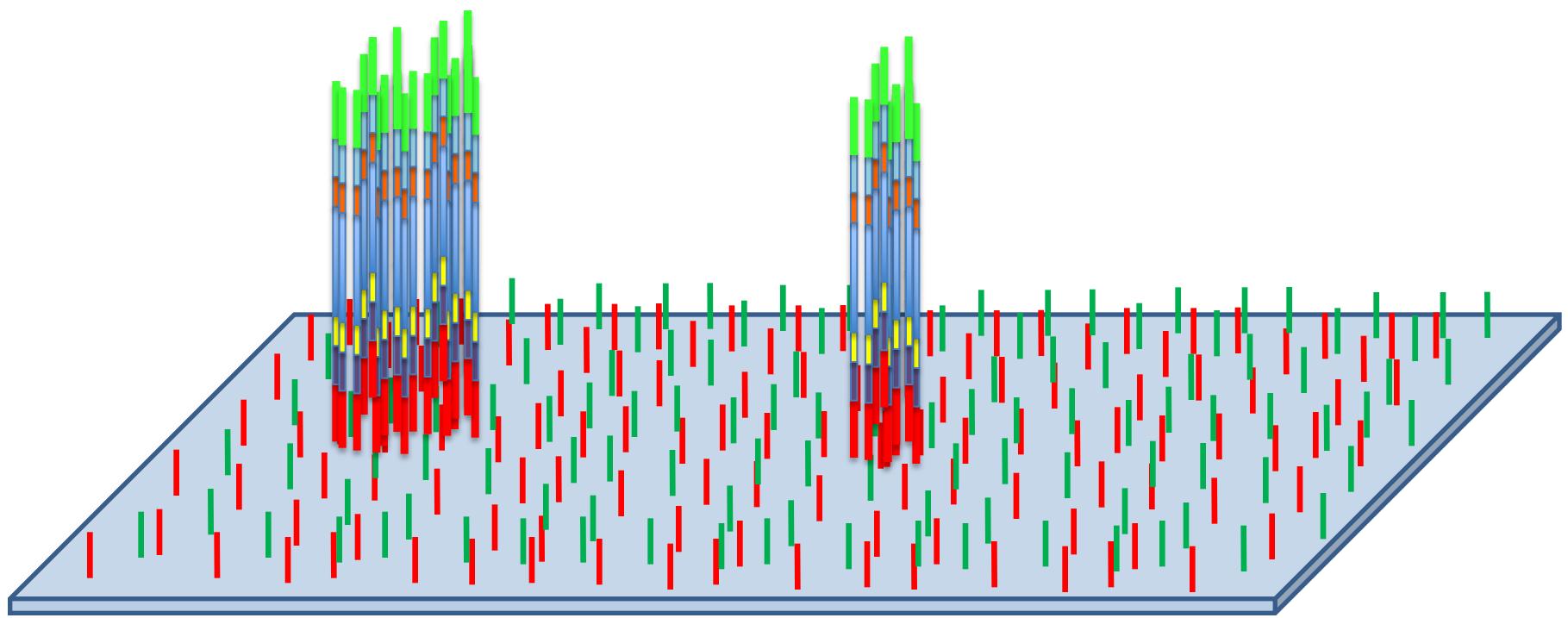


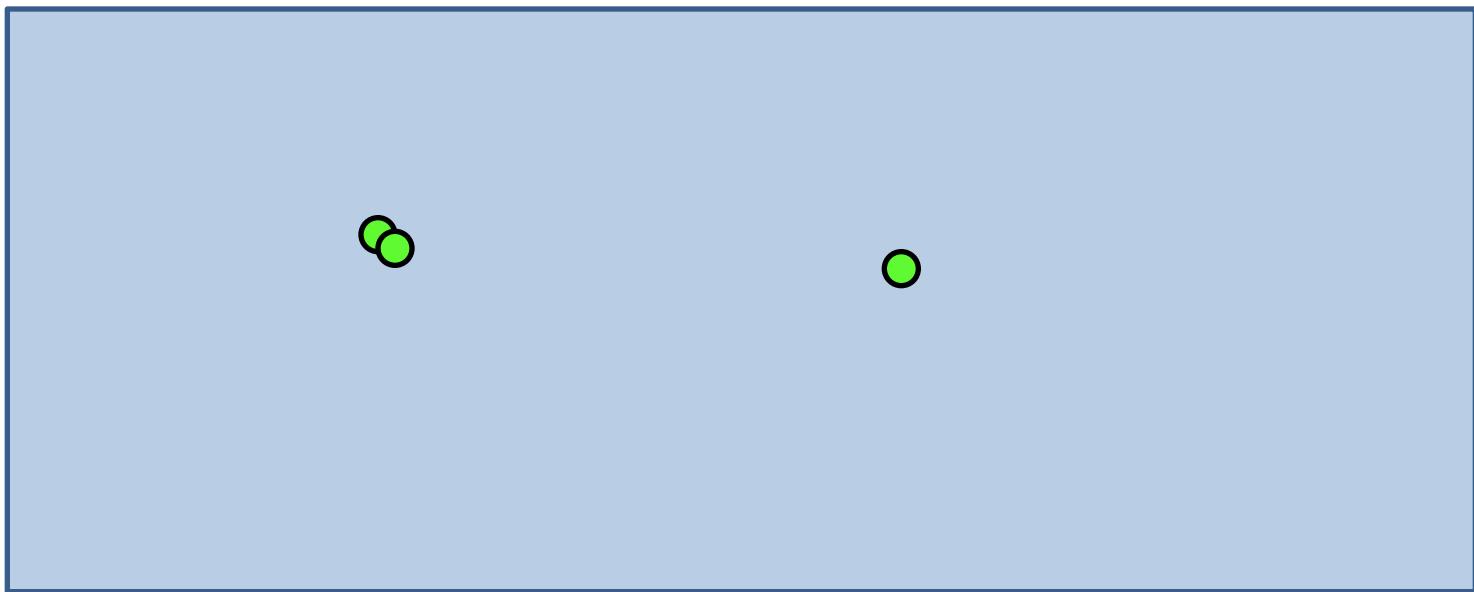


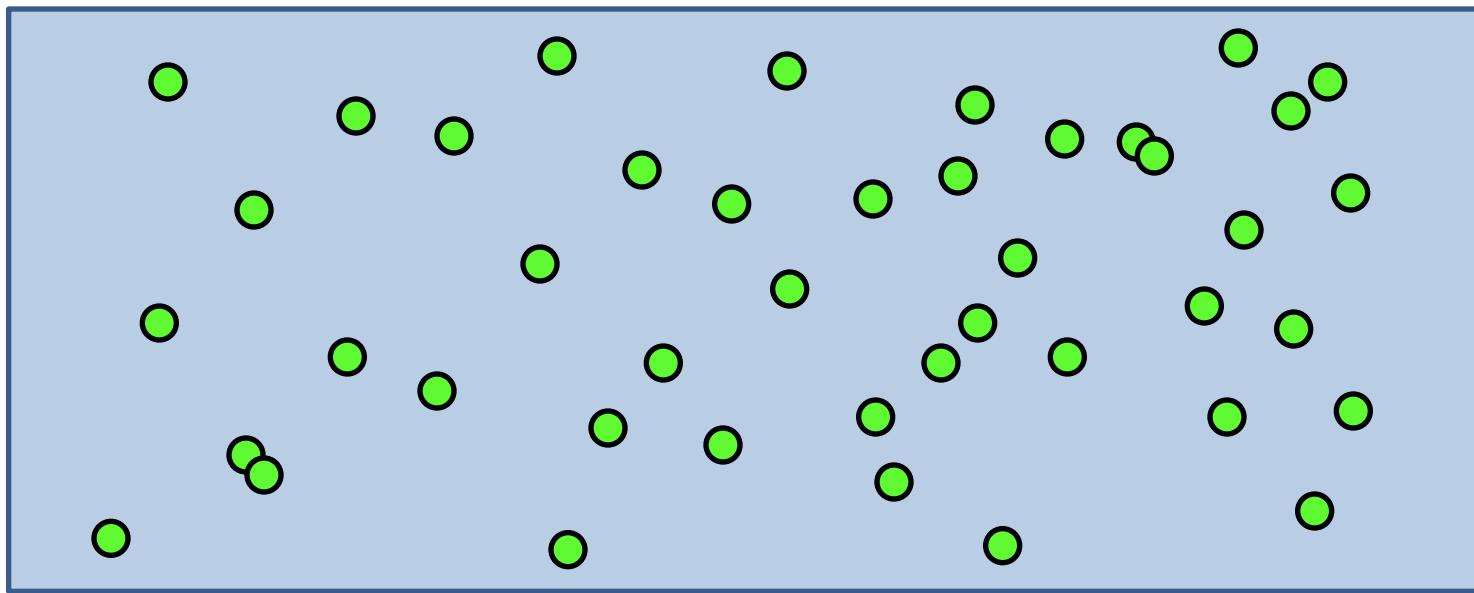




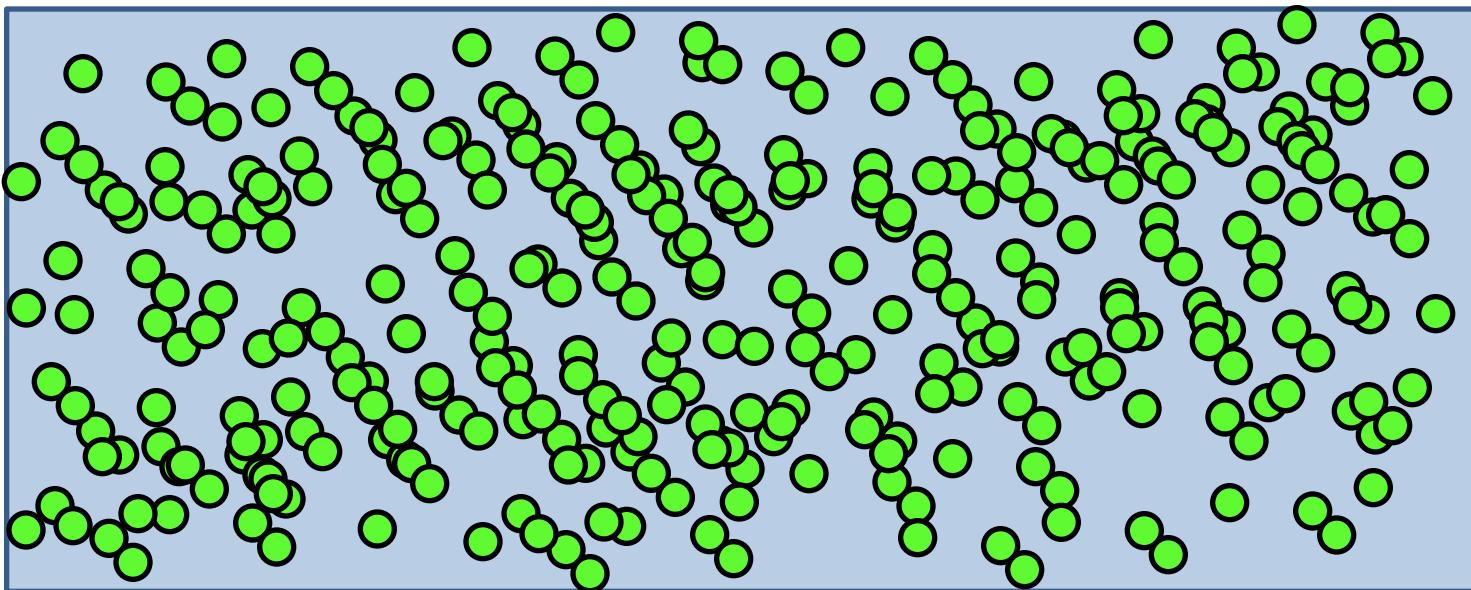


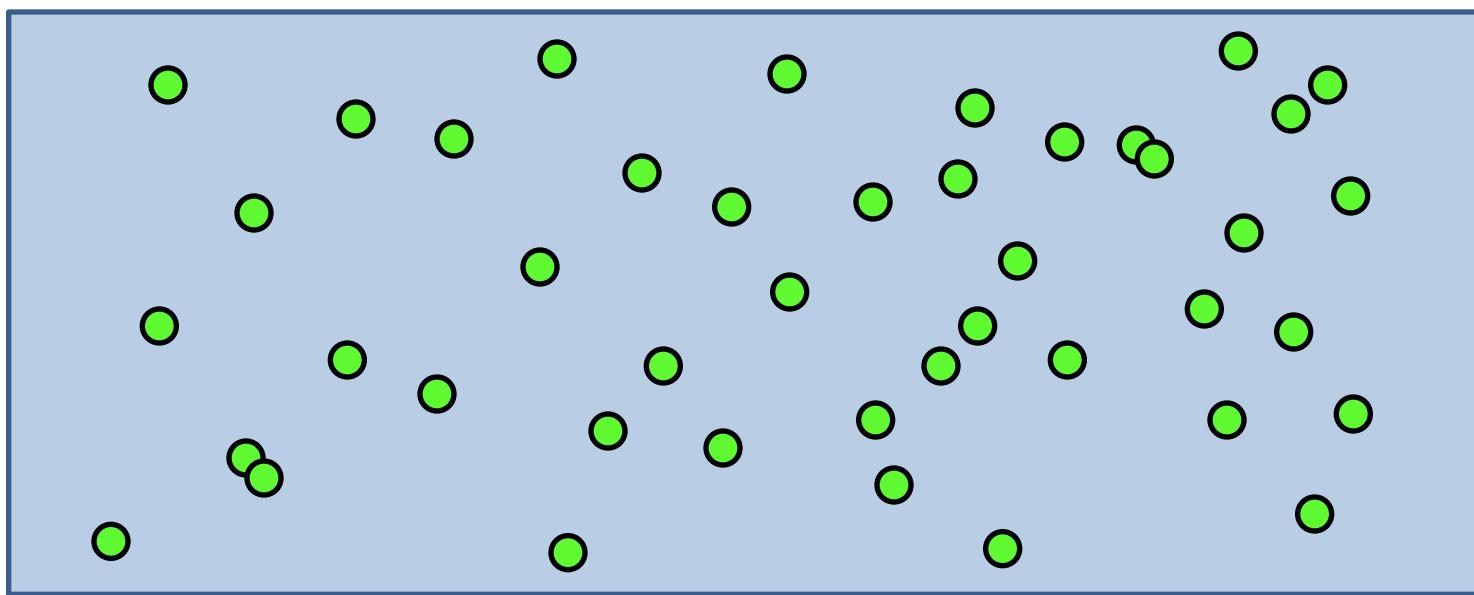




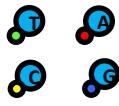


Over - clustering

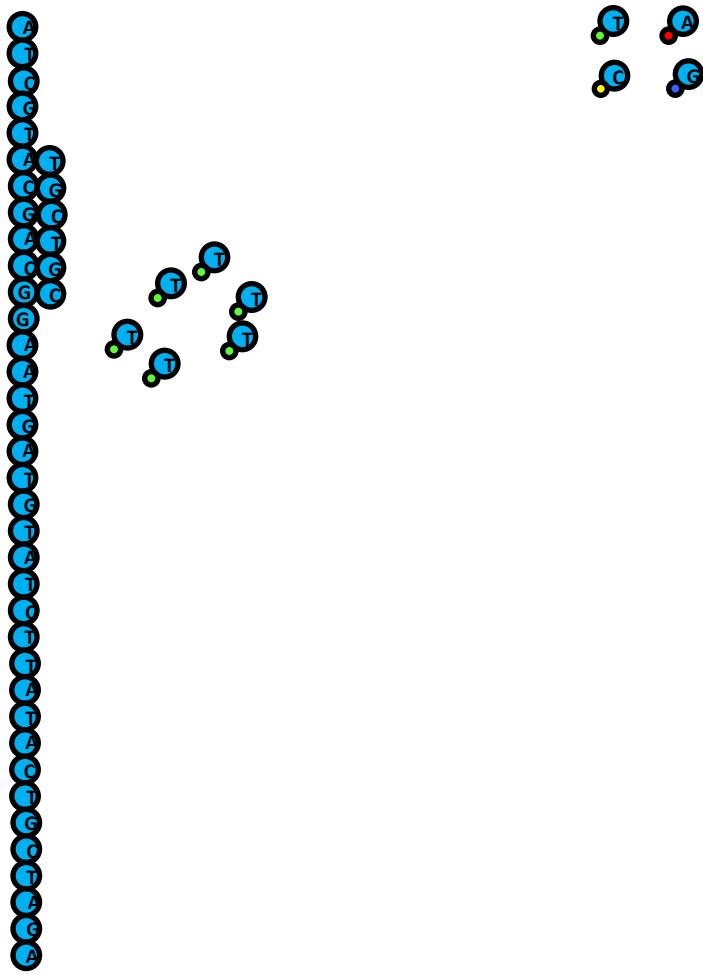




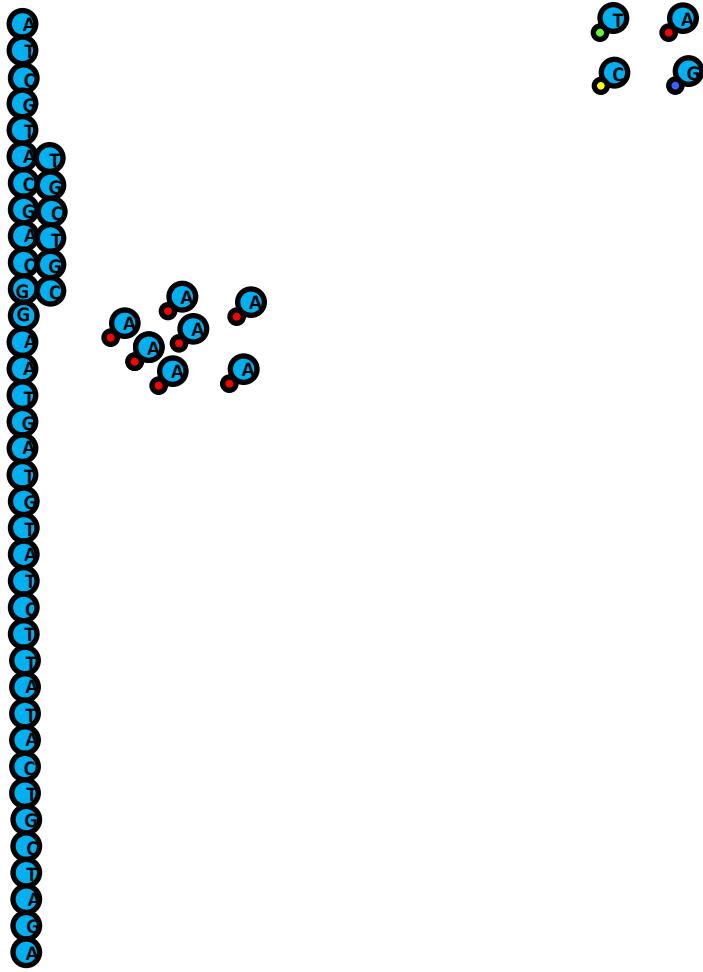
Sequencing by synthesis



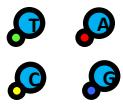
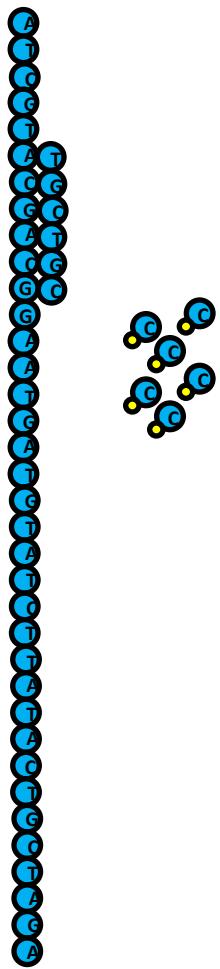
Sequencing by synthesis



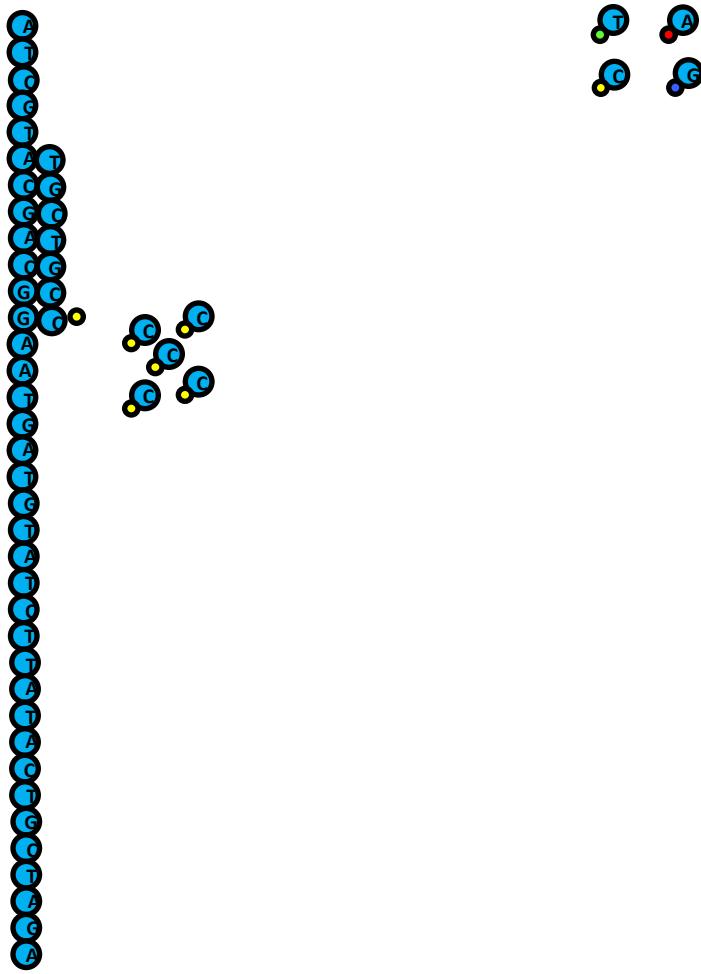
Sequencing by synthesis



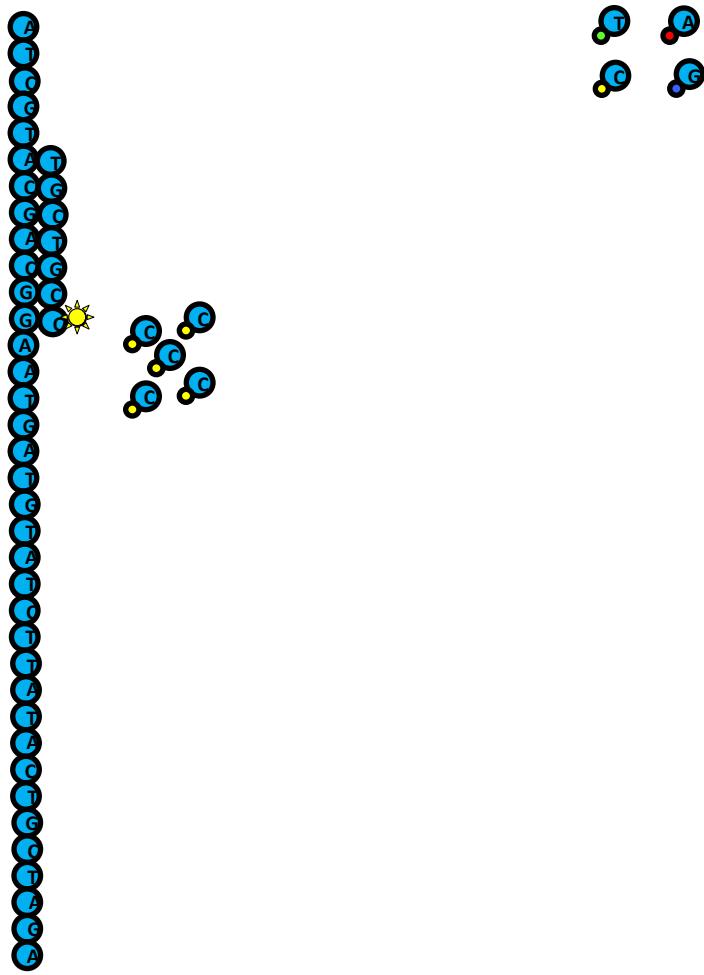
Sequencing by synthesis



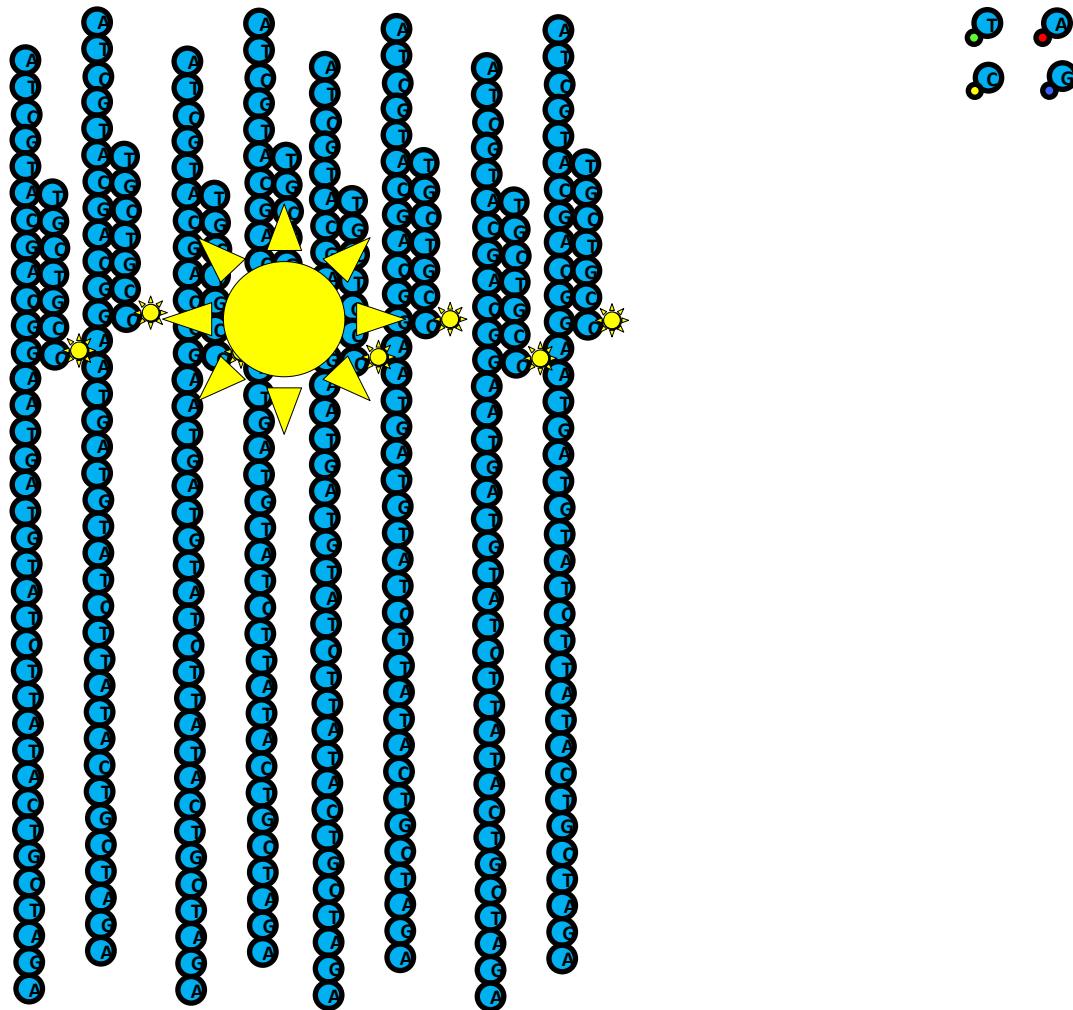
Sequencing by synthesis



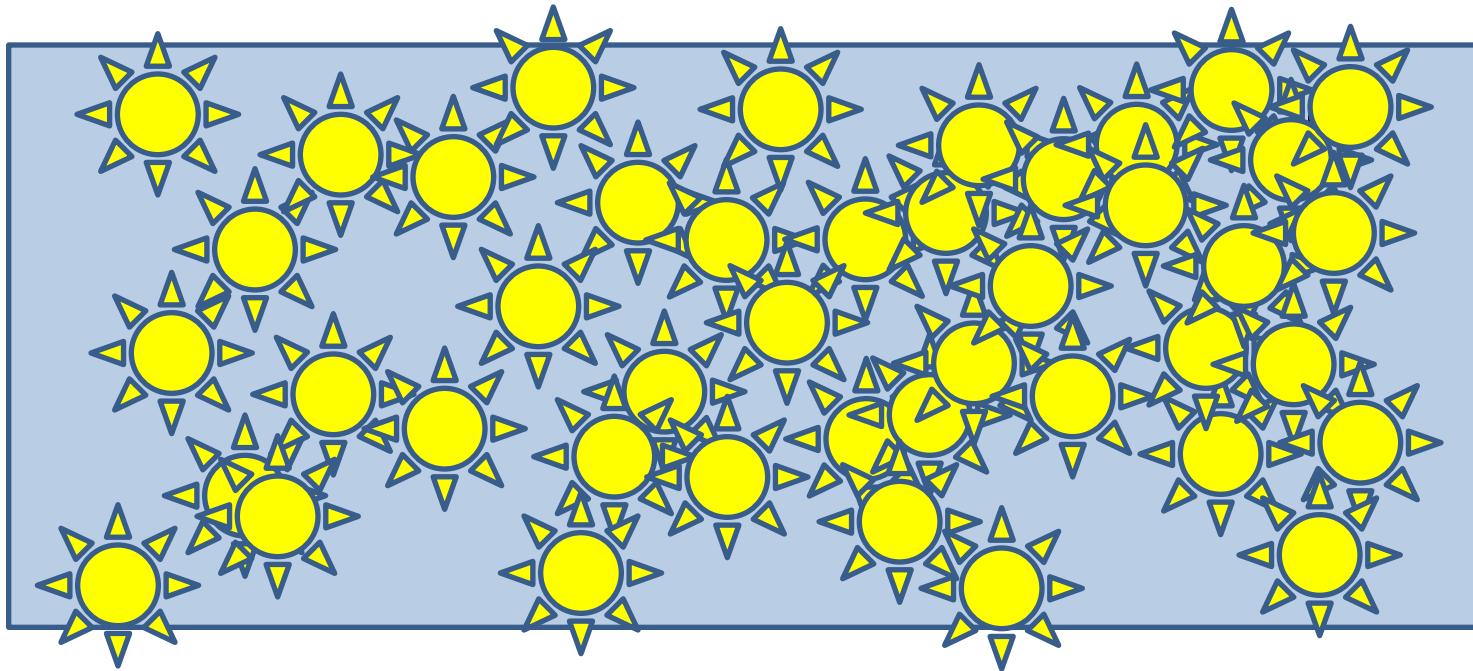
Sequencing by synthesis

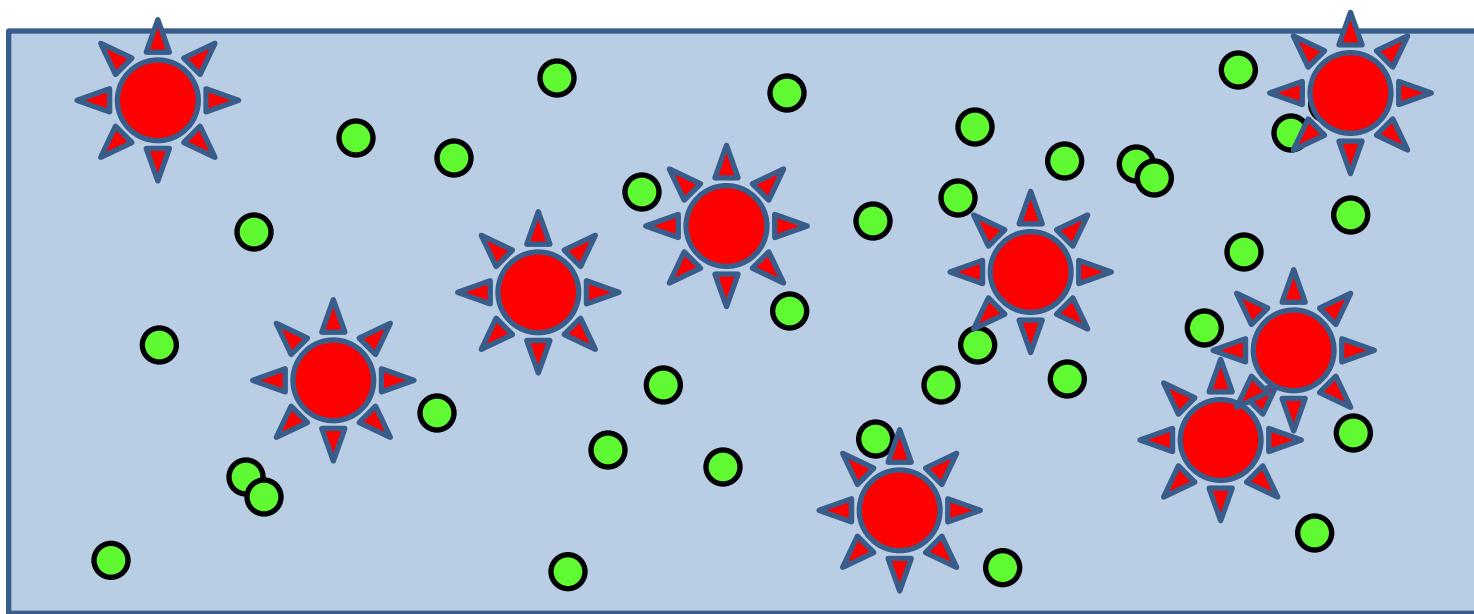


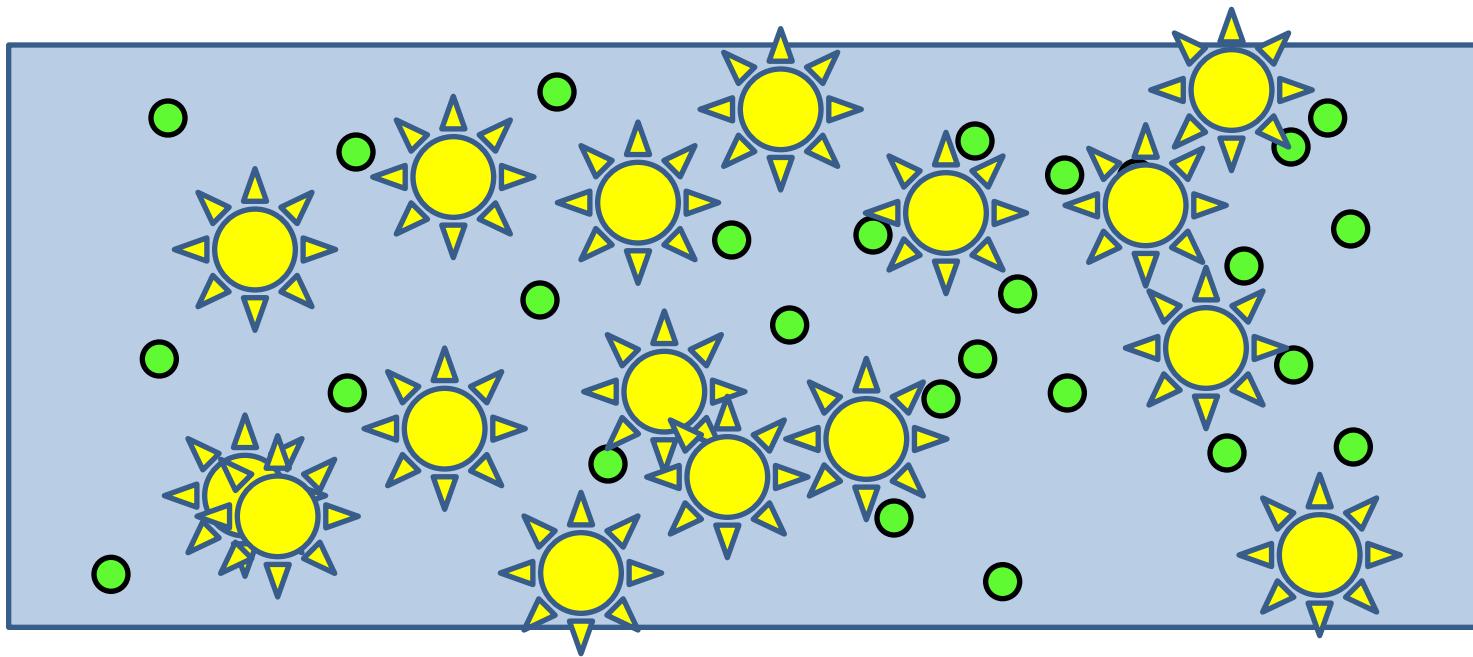
Sequencing by synthesis

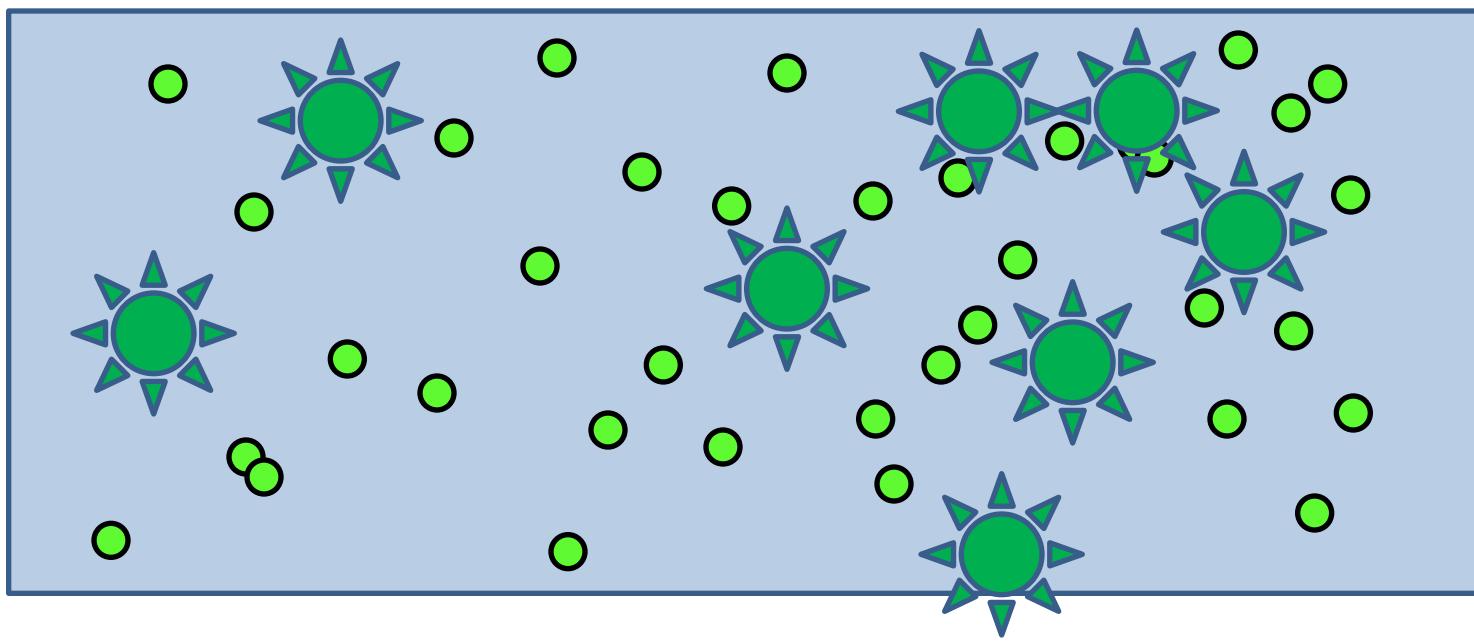


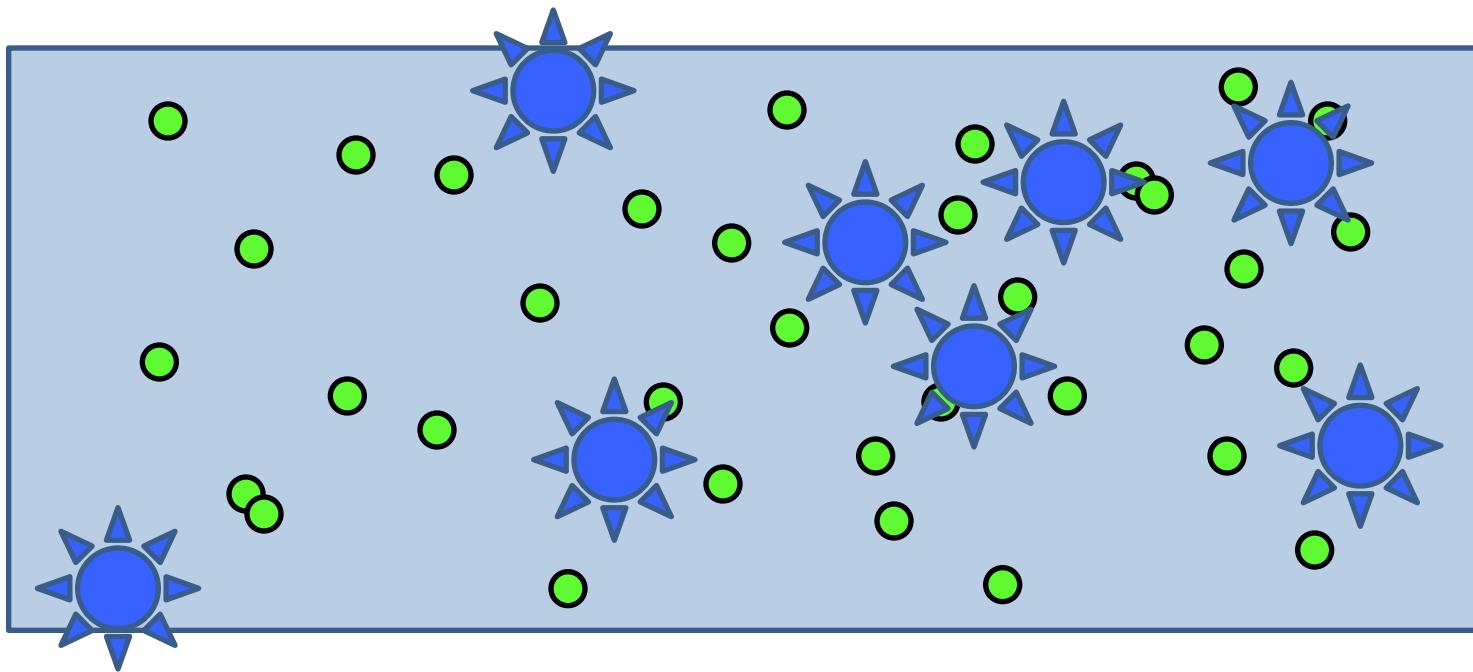
C



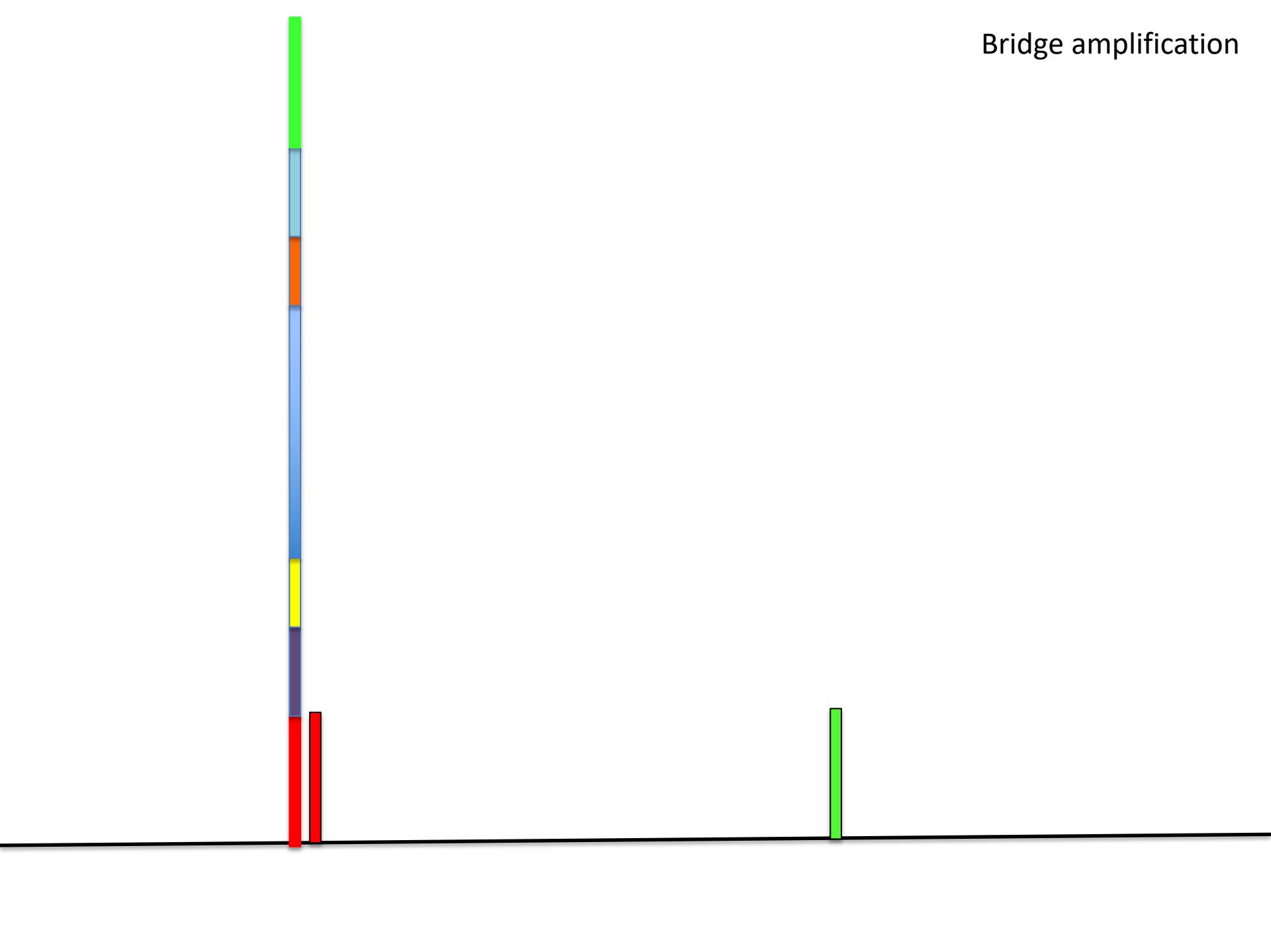




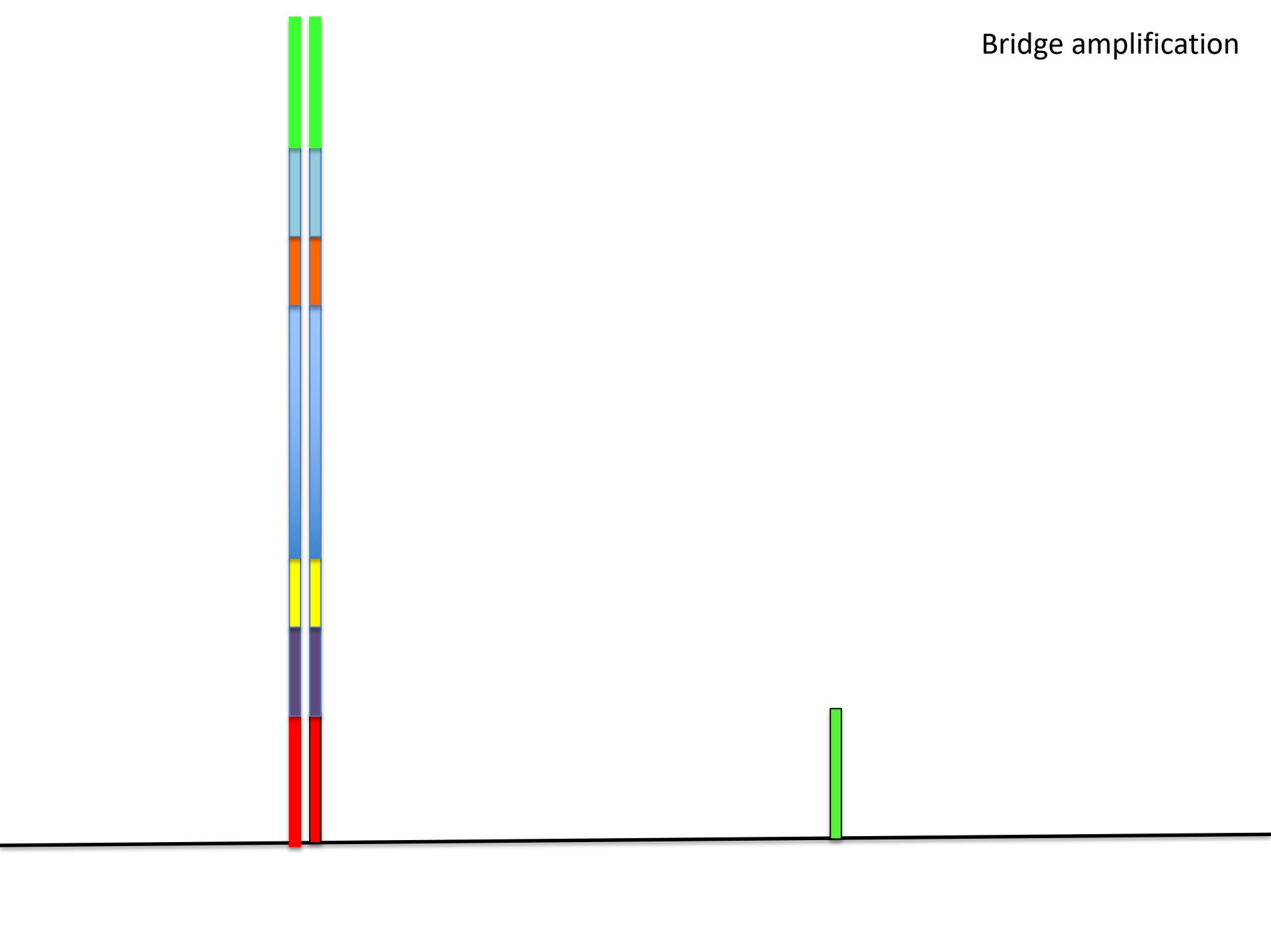




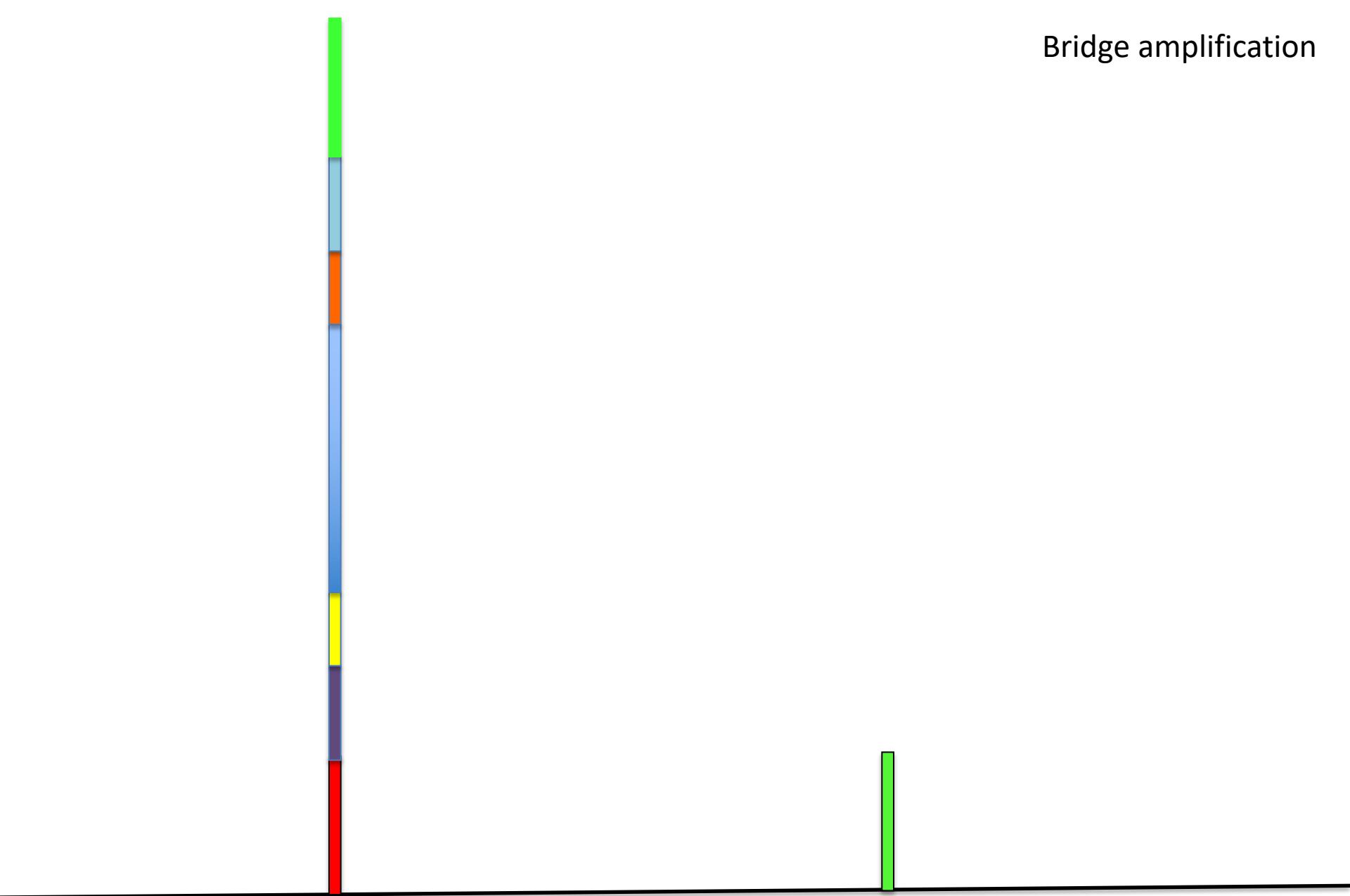
Bridge amplification



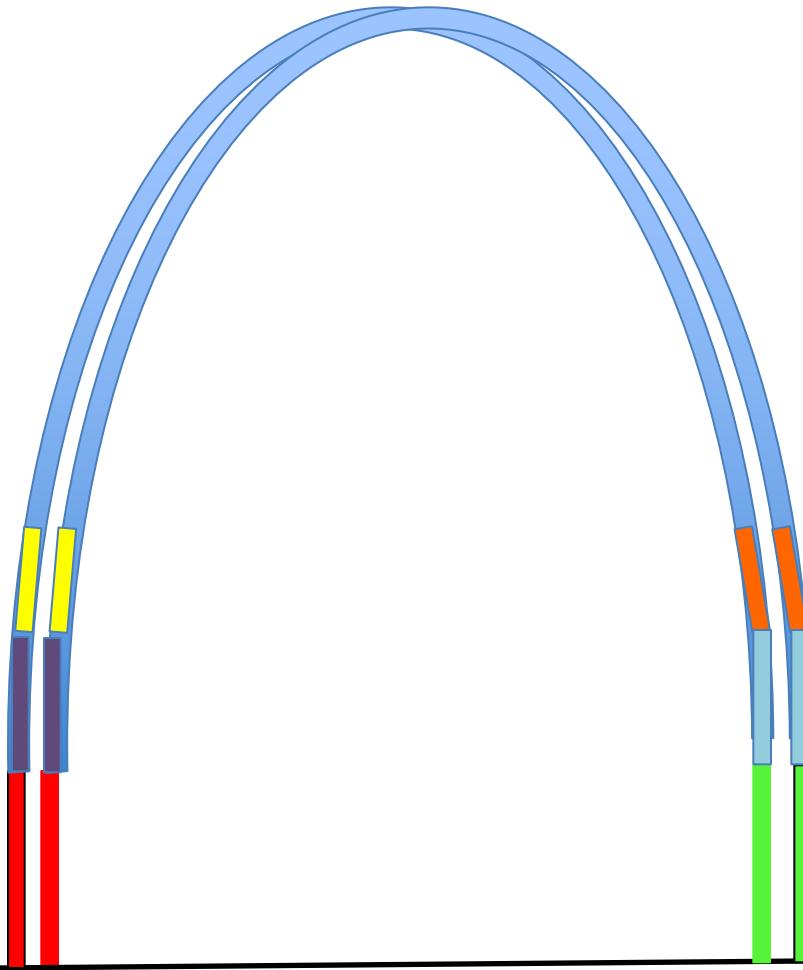
Bridge amplification



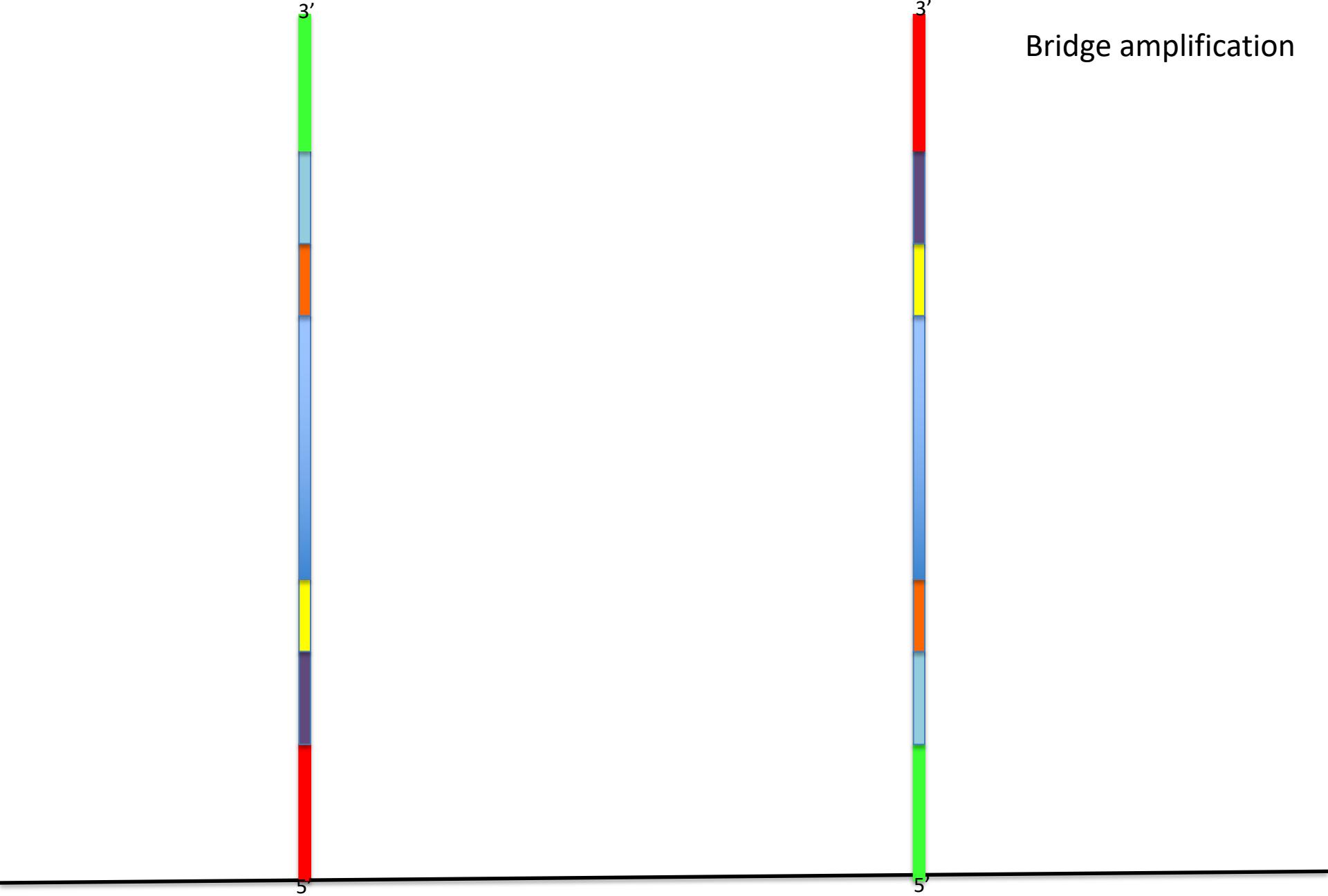
Bridge amplification

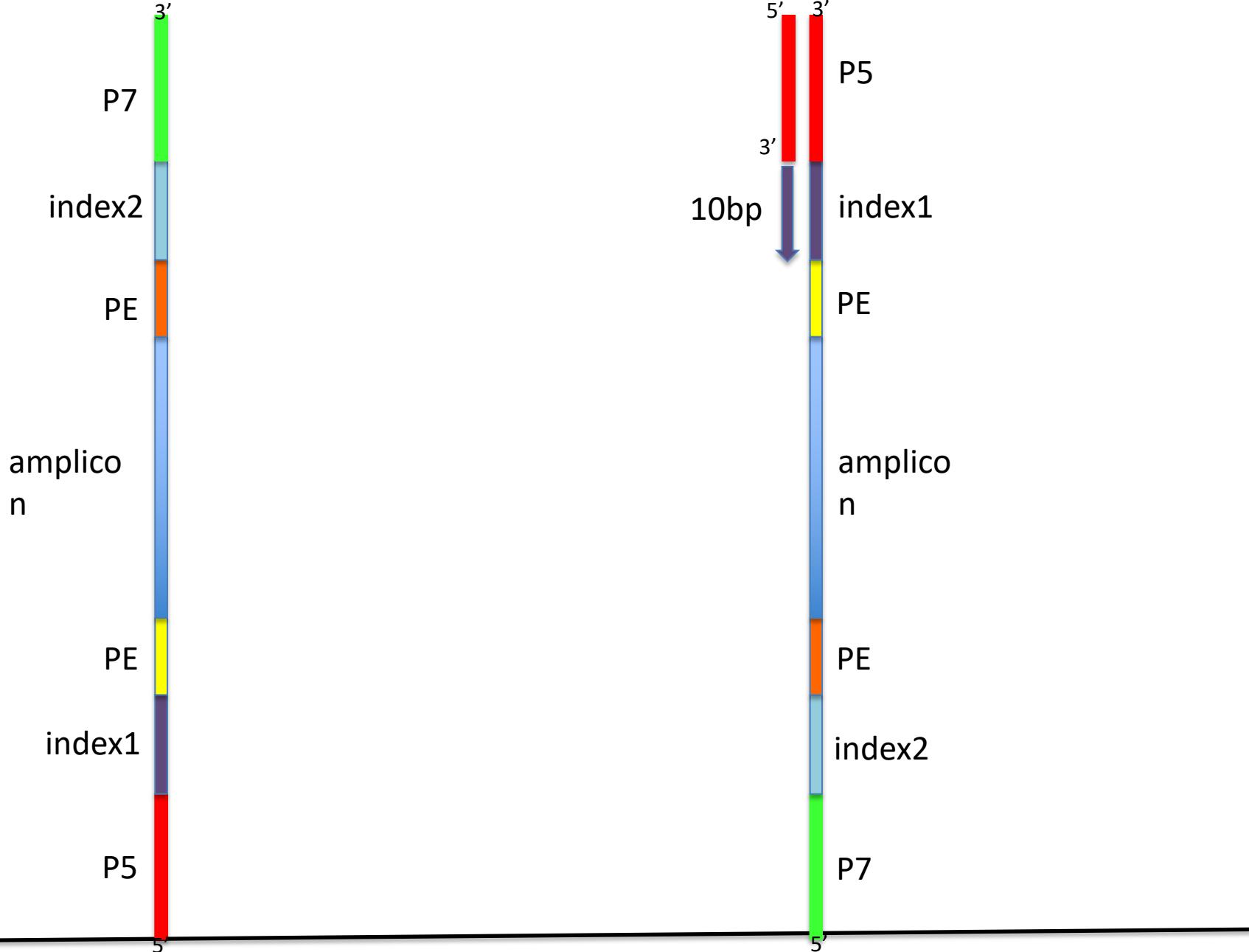


Bridge amplification

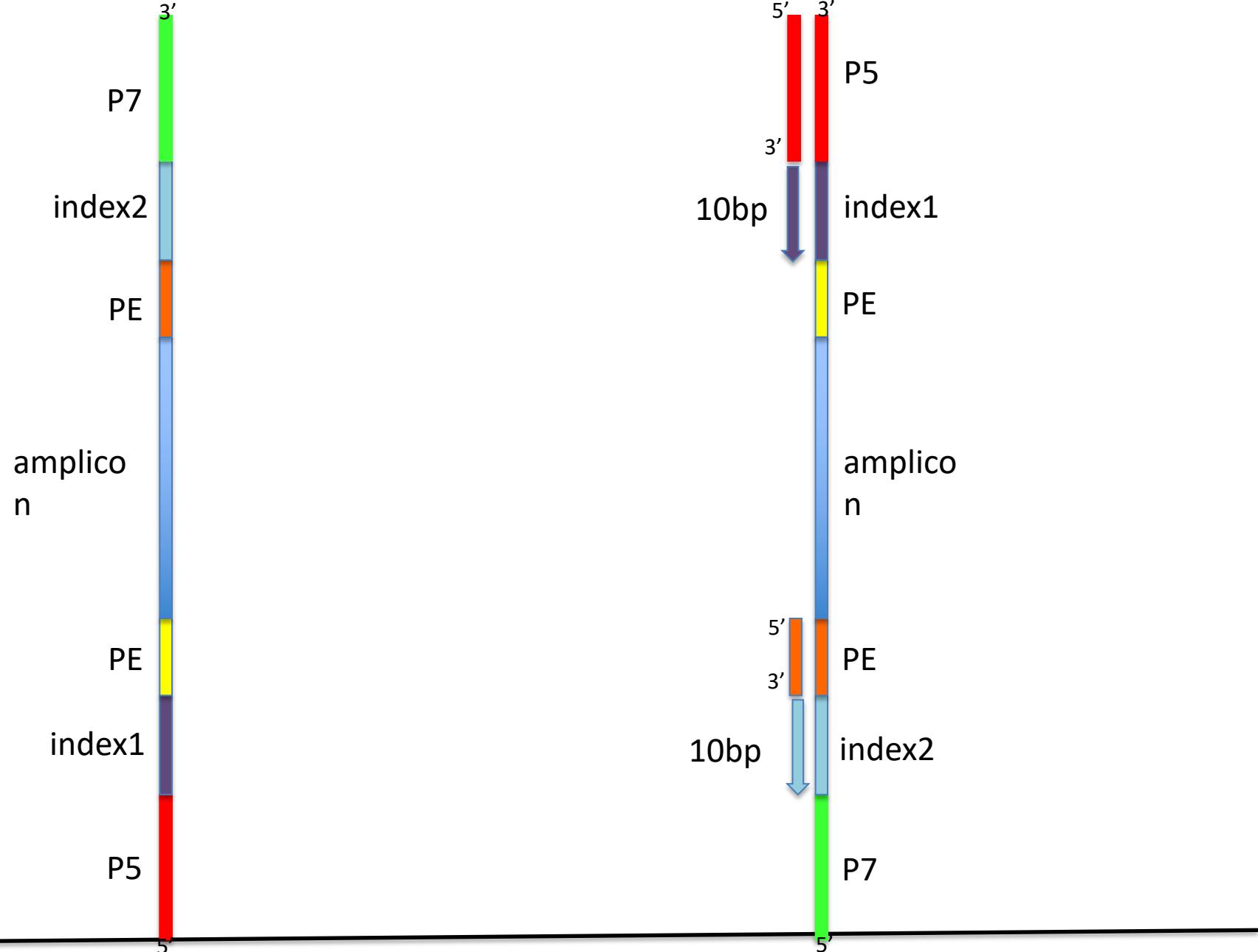


Bridge amplification



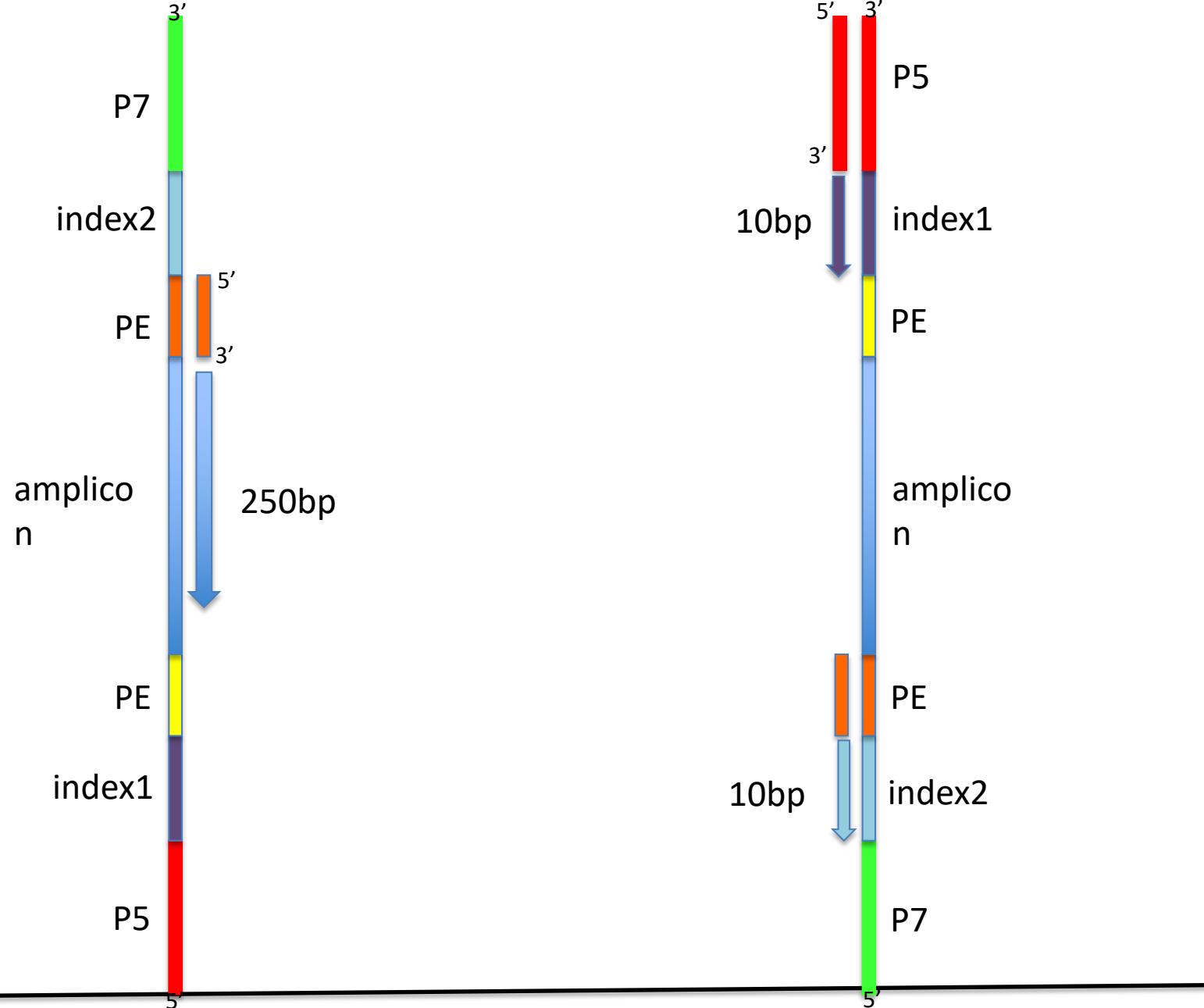


Index 1 sequence: ATCGAC.....



Index 1 sequence: ATCGAC.....

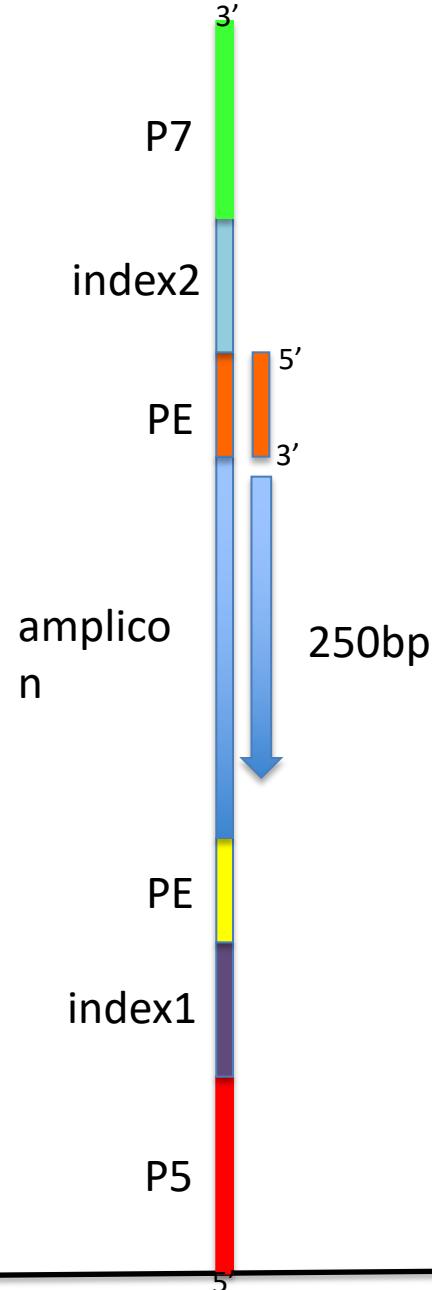
Index 2 sequence: GGCTATC.....



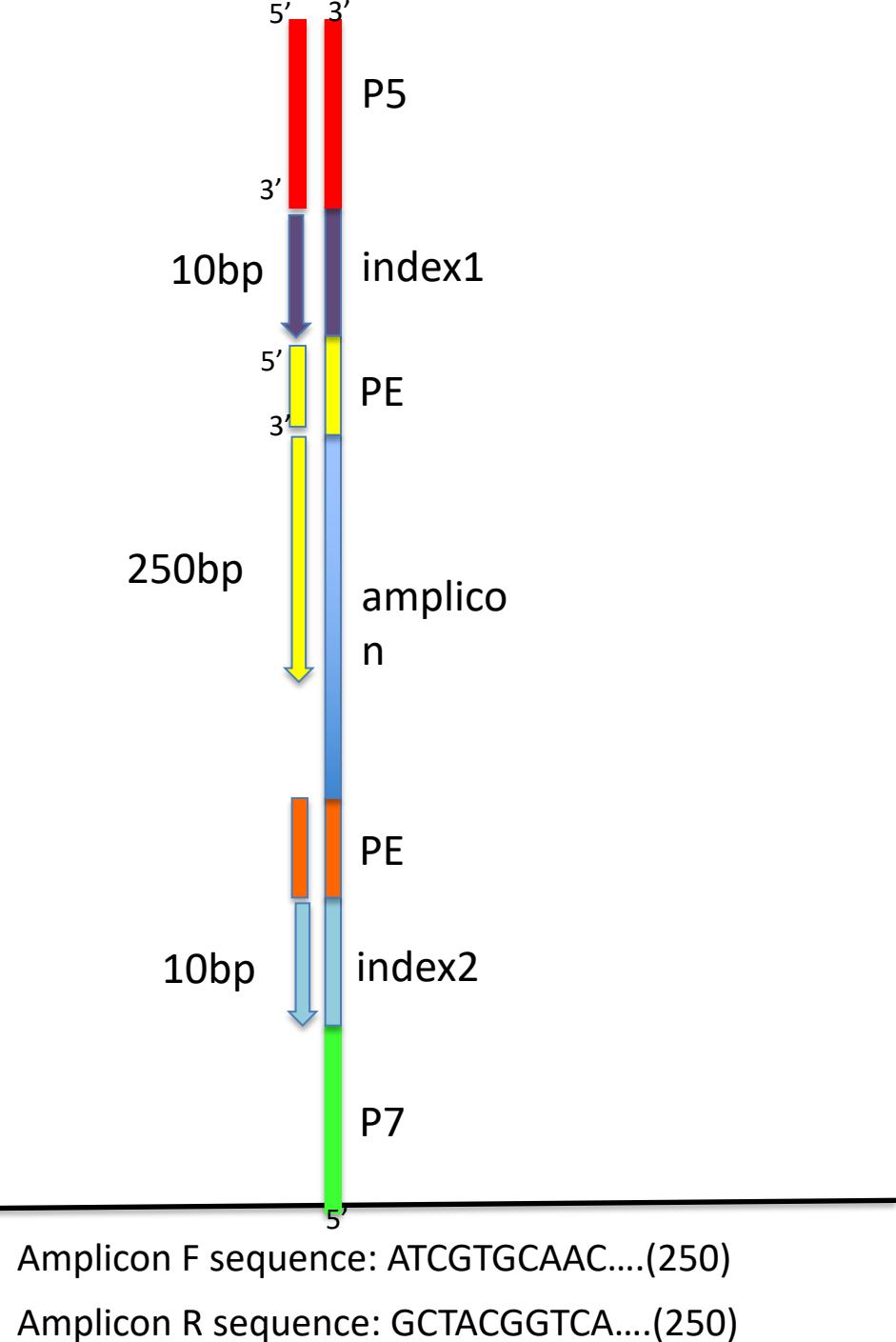
Index 1 sequence: ATCGAC.....

Index 2 sequence: GGCTATC.....

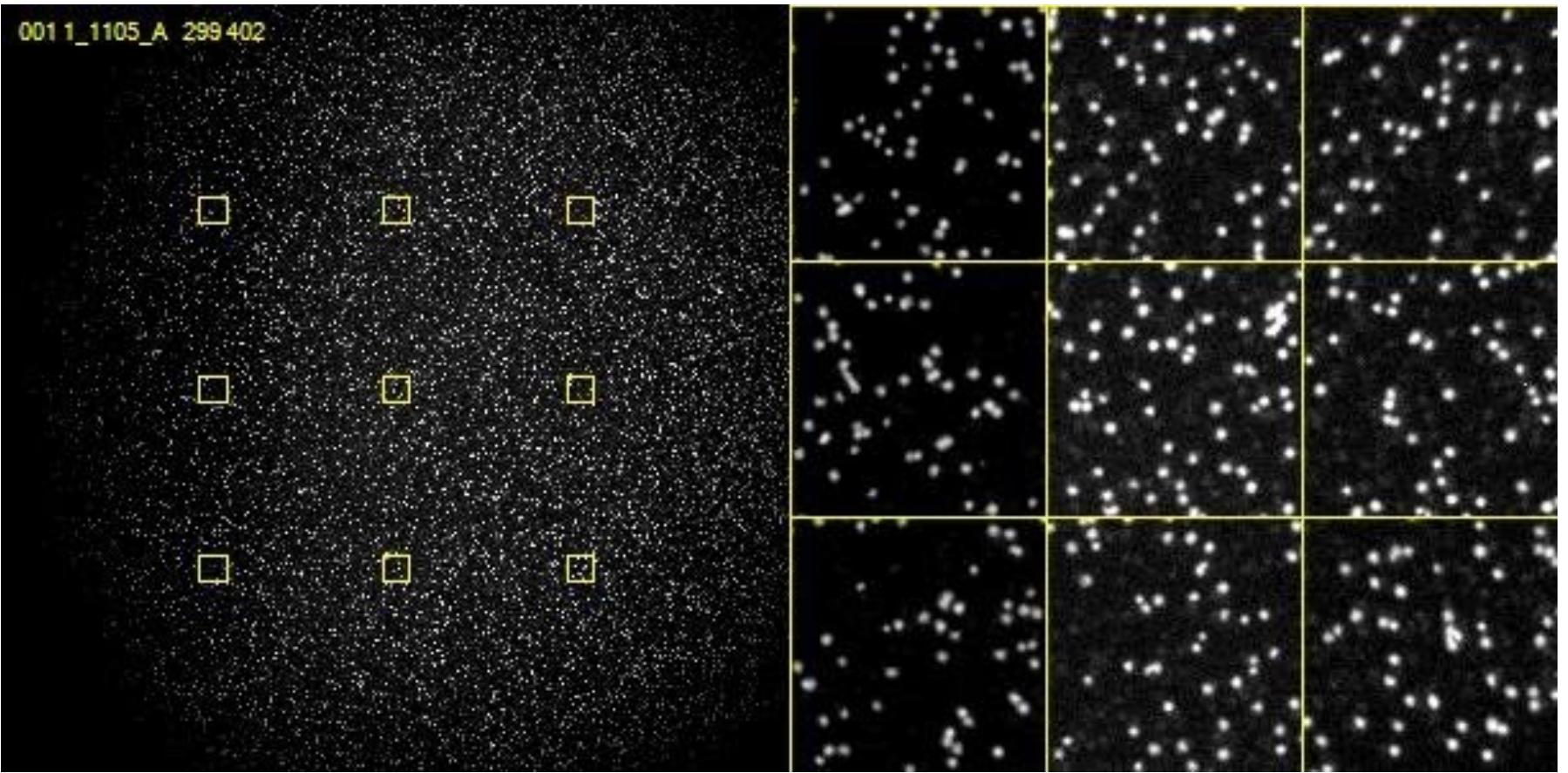
Amplicon F sequence: ATCGTGCAAC....(250)



Index 1 sequence: ATCGAC.....
Index 2 sequence: GGCTATC.....



Amplicon F sequence: ATCGTGCAAC....(250)
Amplicon R sequence: GCTACGGTCA....(250)





Miniseq System

MAX OUTPUT
8 Gb

MAX READ NUMBER
25 million

MAX READ LENGTH
2x150 bp



MiSeq Series

MAX OUTPUT
15 Gb

MAX READ NUMBER
25 million

MAX READ LENGTH
2x300 bp



NextSeq System

MAX OUTPUT
120 Gb

MAX READ NUMBER
400 million

MAX READ LENGTH
2x150 bp



HiSeq Series

MAX OUTPUT
1500 Gb

MAX READ NUMBER
5 billion

MAX READ LENGTH
2x150 bp



HiSeq X Series

MAX OUTPUT
1800 Gb

MAX READ NUMBER
6 billion

MAX READ LENGTH
2x150 bp

2nd generation sequencing (Illumina)

Advantages

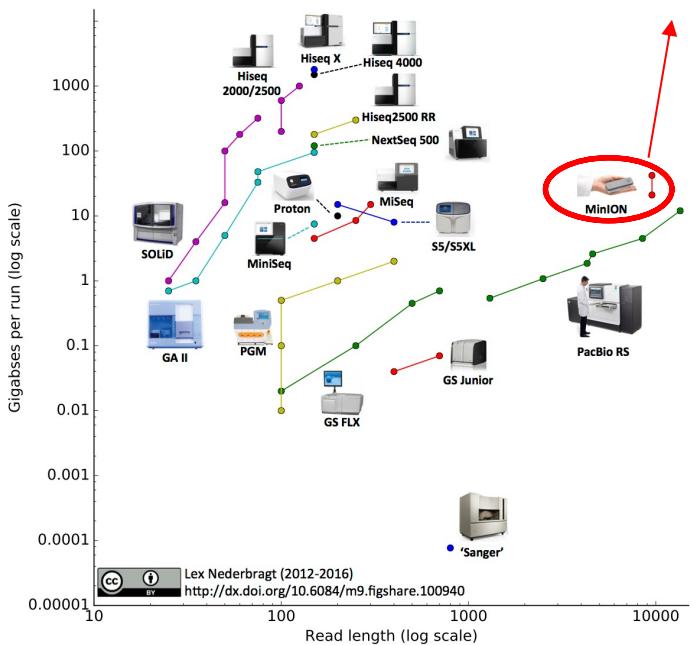
- High throughput
- Lowest cost/base pair
- High quality
- Multiple applications

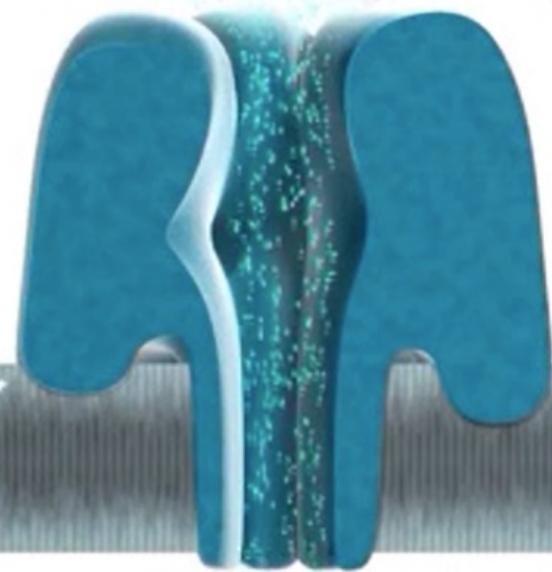
Disadvantages

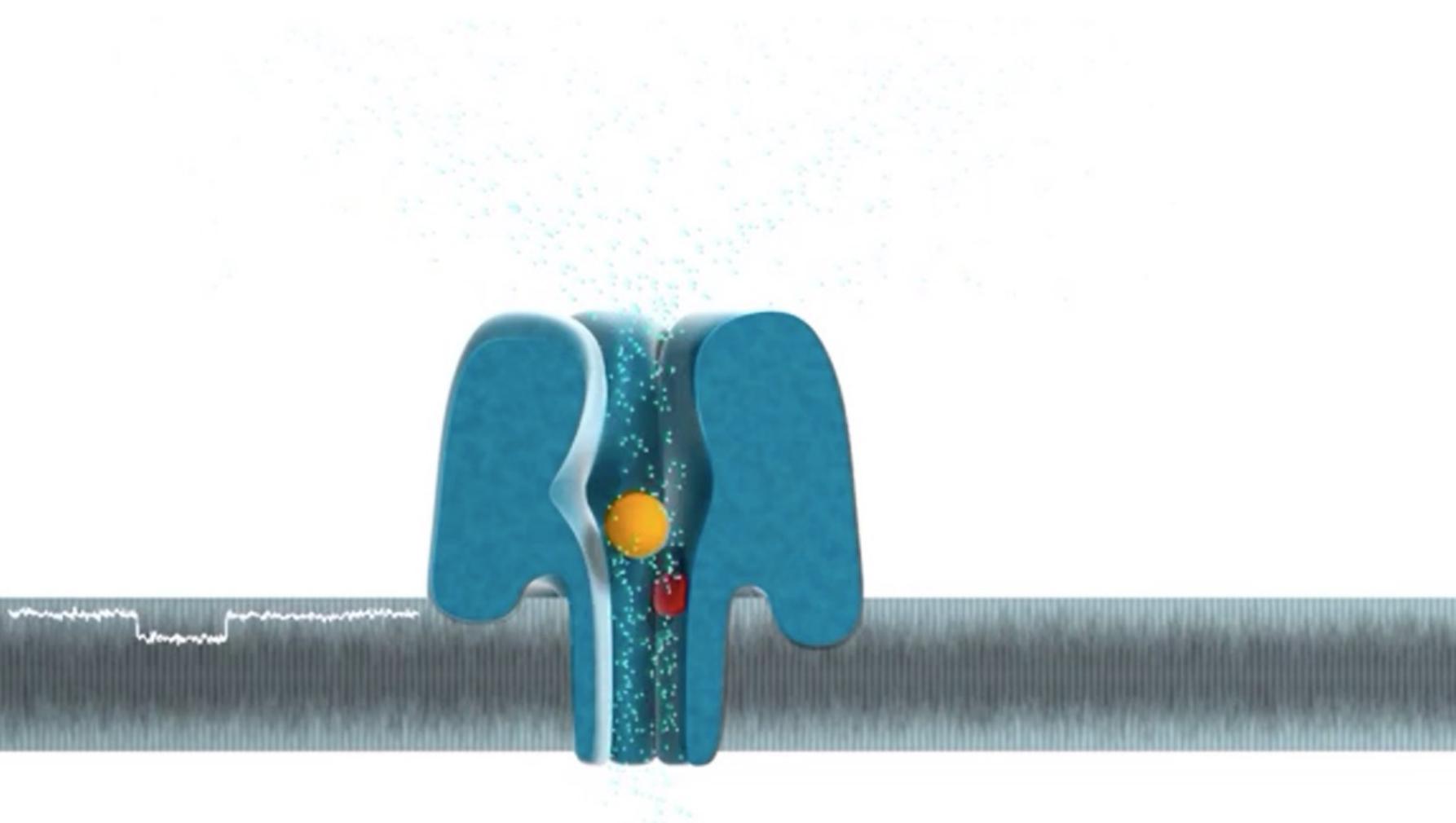
- Short reads
- Tedious library preparation
- Long runtime

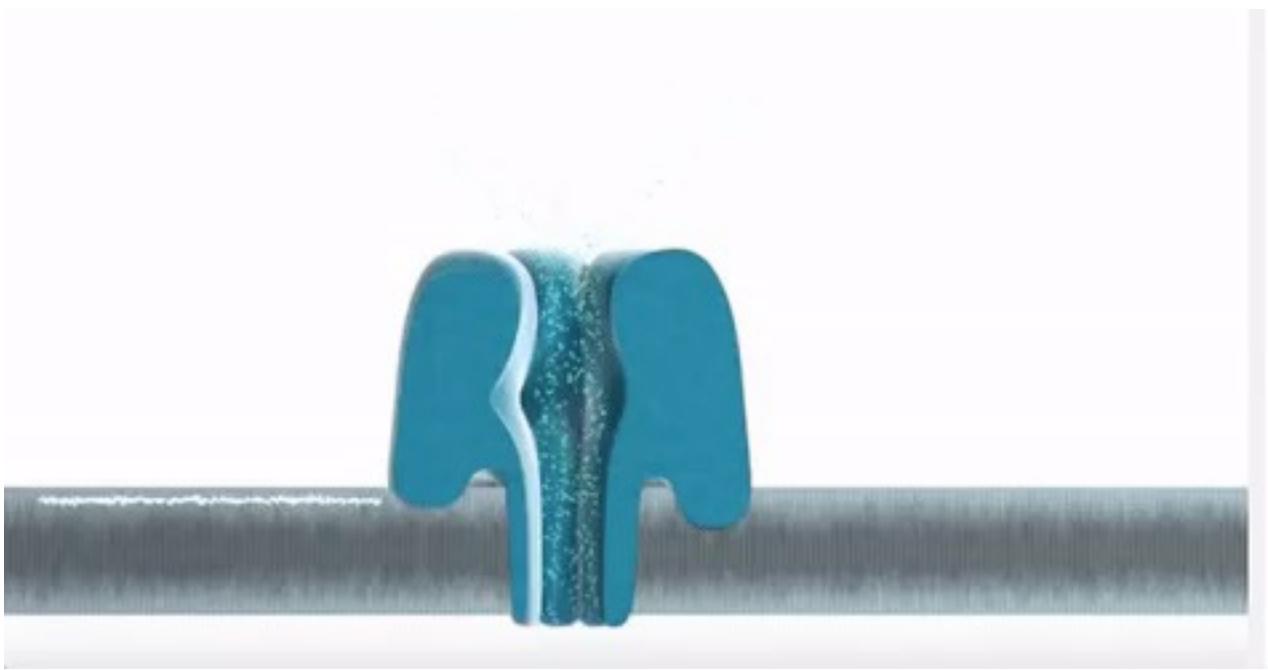
4th Generation Sequencing



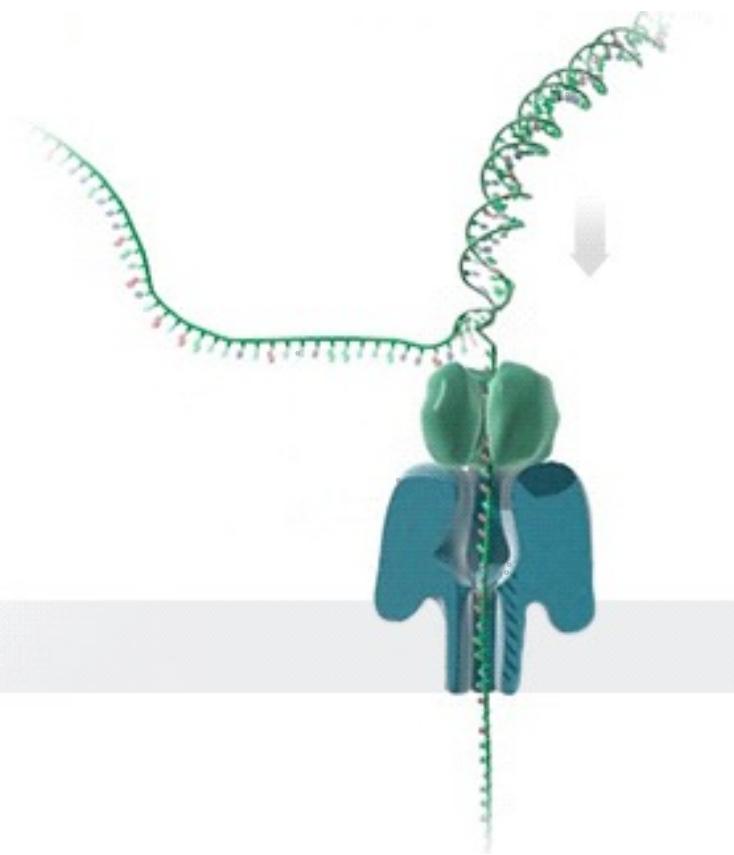


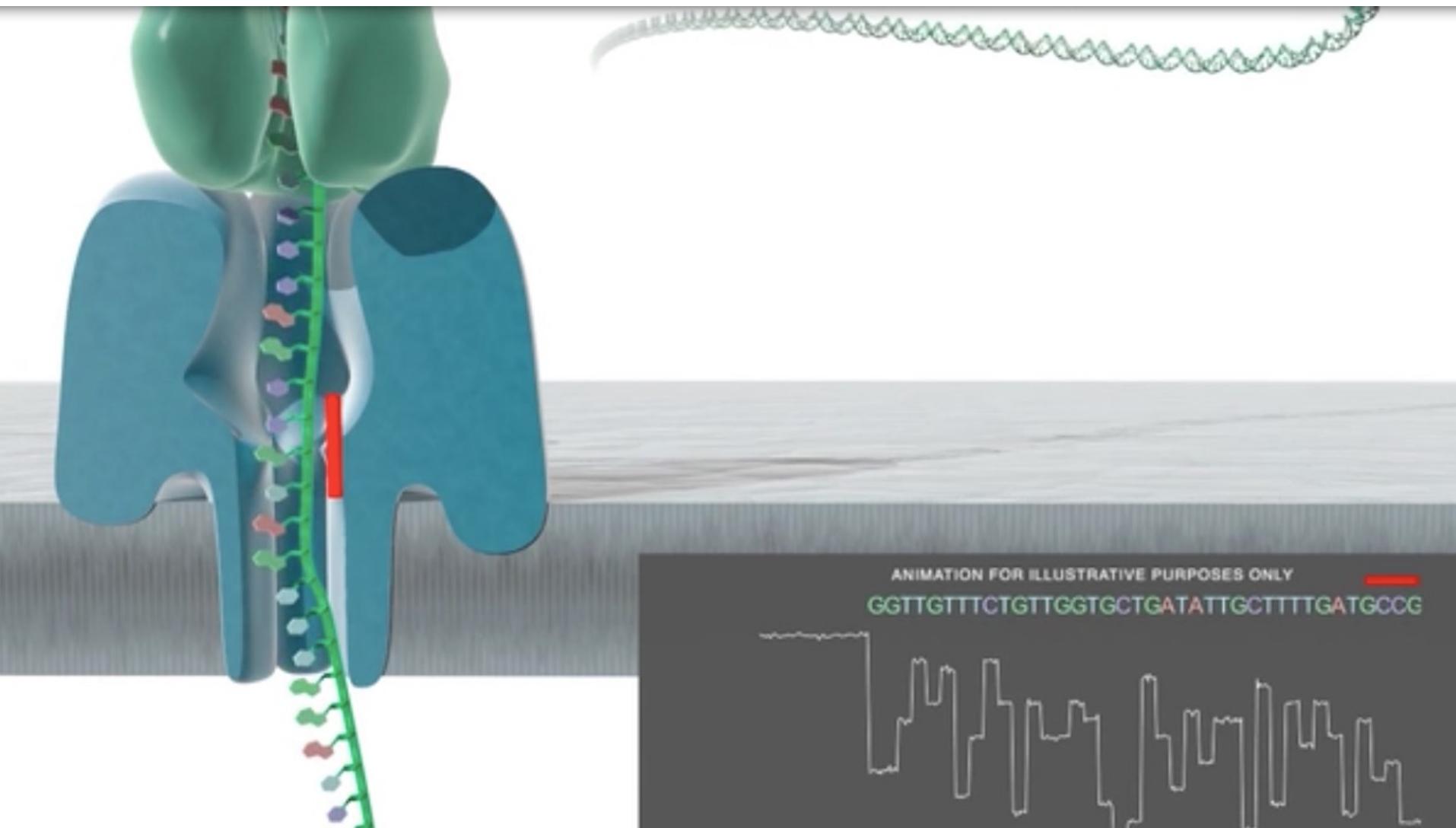






KP







ANIMATION FOR ILLUSTRATIVE PURPOSES ONLY





4rd generation sequencing (Oxford Nanopore)

Real time sequencing without fluorescence chemistry

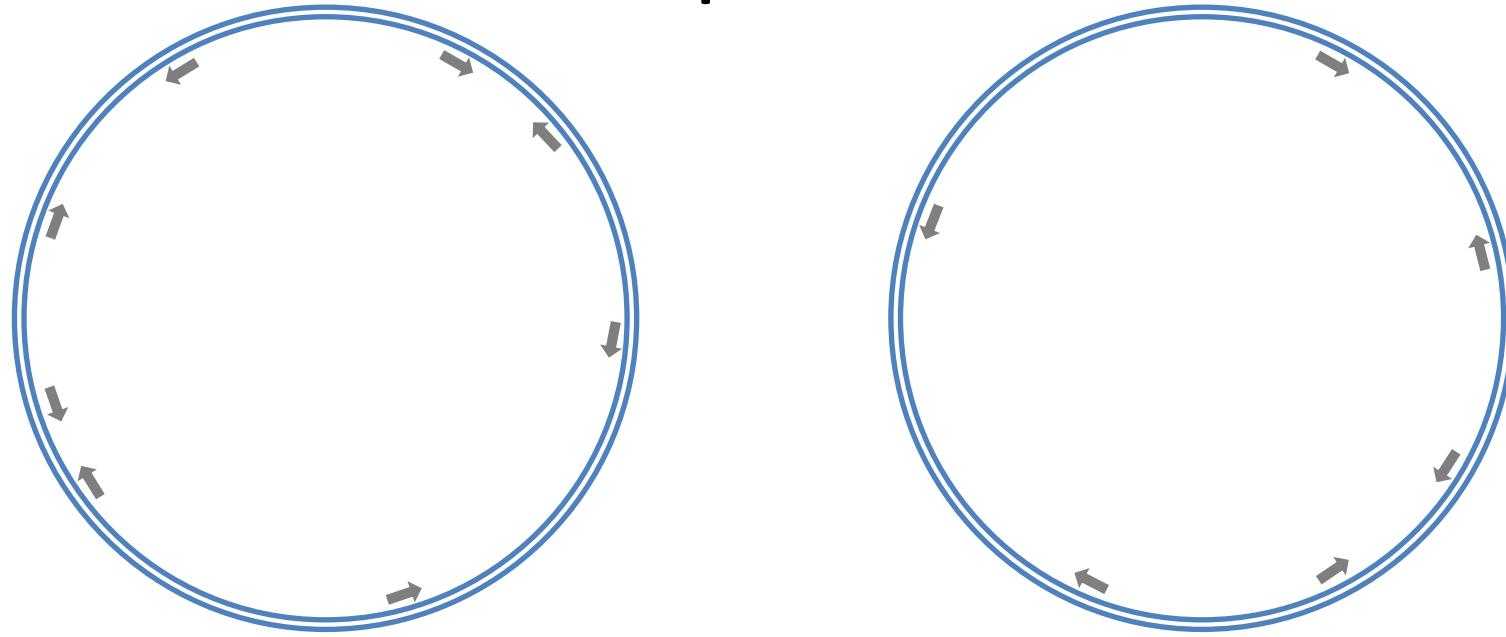
Advantages

- Long reads
- Fast runtime
- Potentially high throughput (modular design)
PromethION (144,000 nanopore sensors)

Disadvantages

- High error rate 38% at the beginning of 2015
- About 5% in 2016... e.g. Illumina about 1%

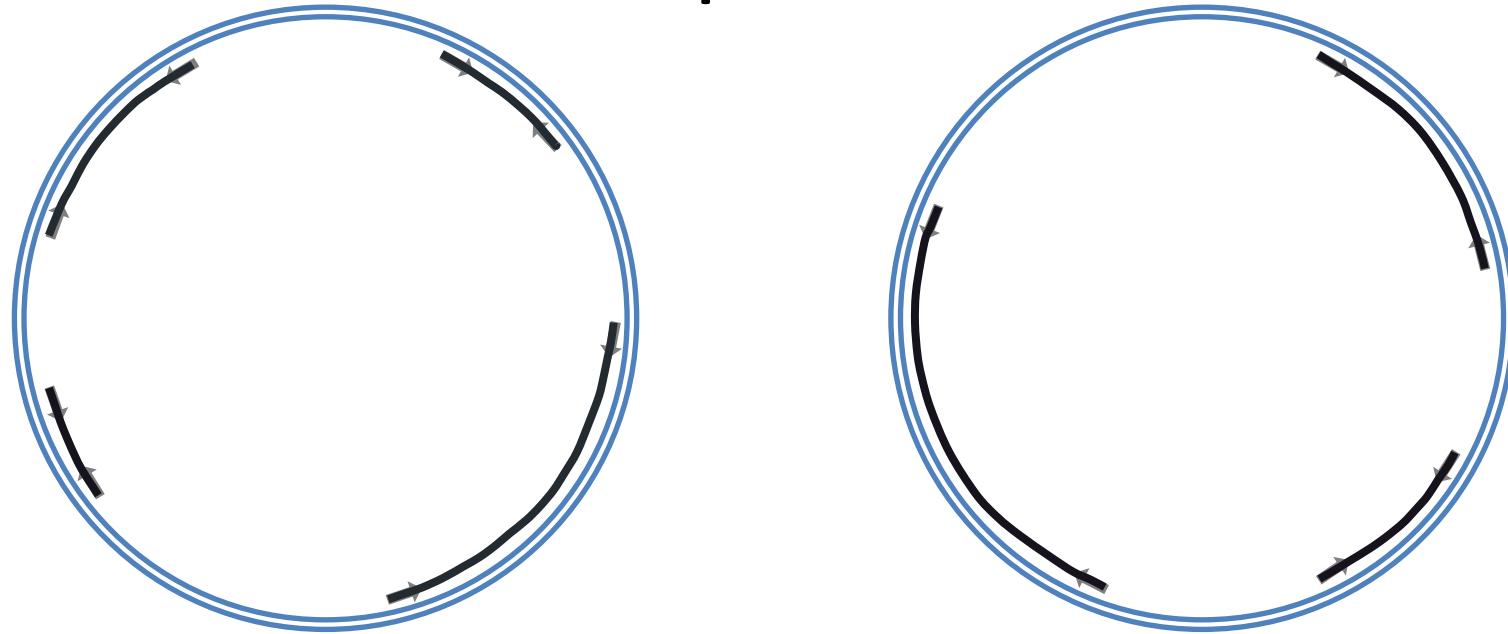
Rep-PCR



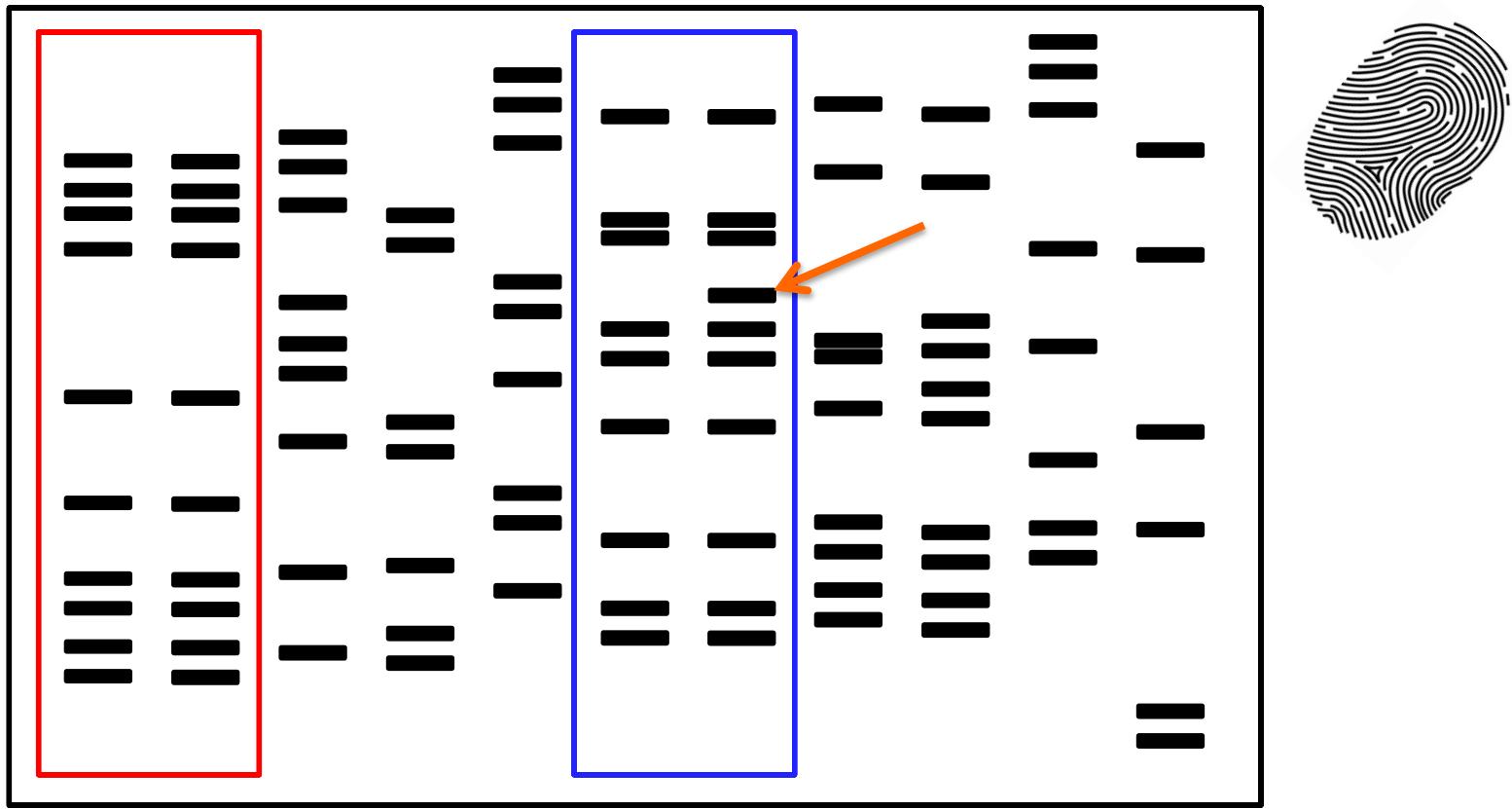
Single primer (GTG)5

Targeting repetitive palindromic sequences in bacterial genomes

Rep-PCR

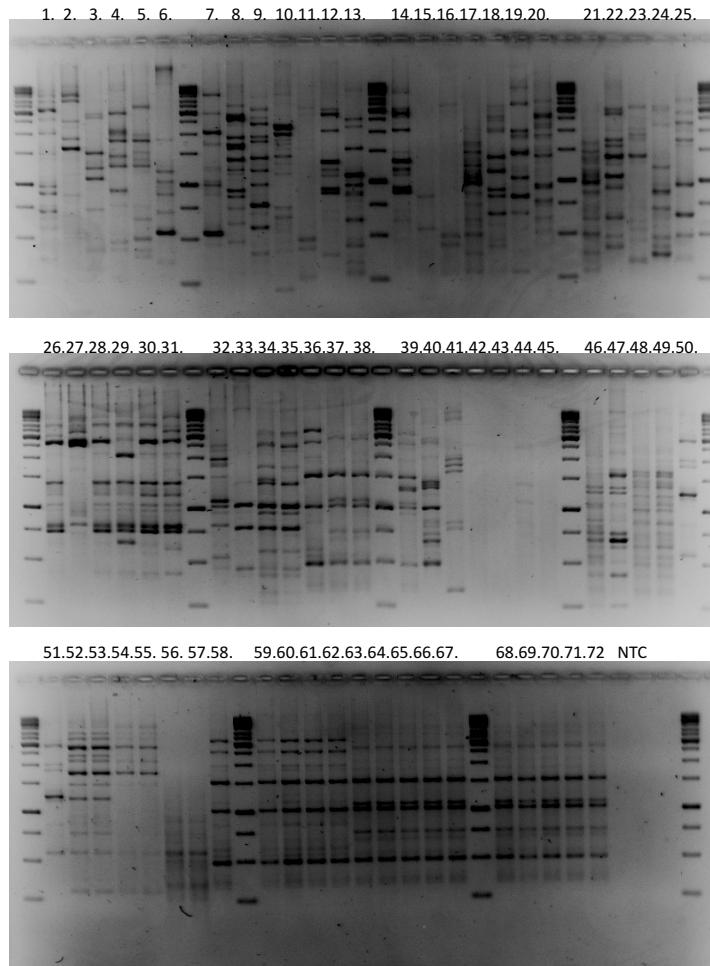


Amplicons varying in amount and size unique to given bacterial species/strain are being generated



In general, closely related bacteria will generate similar fingerprint profiles.
e.g: two different strains of the same bacterial species might differ with very few bands

Laborious ...

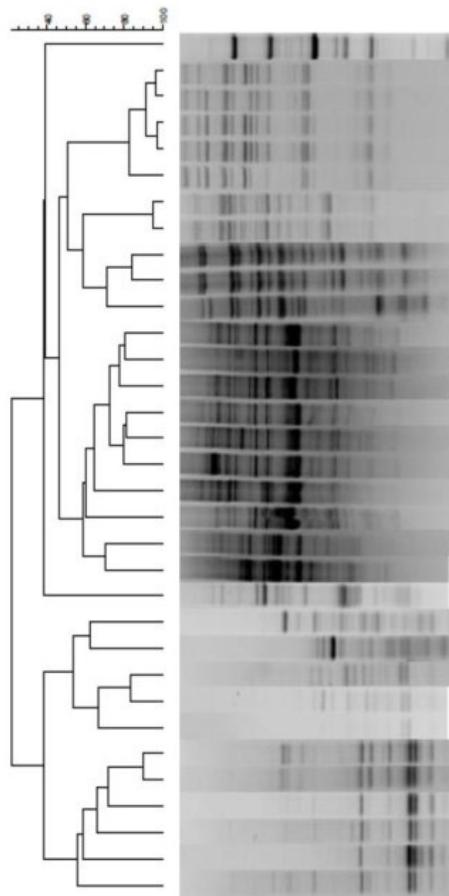


The method is highly laborious, as each isolate needs to be loaded on gel, multiple gels need to be prepared and similar conditions and quality needs to be provided to ensure that results are comparable

Limited information

So why bother ?

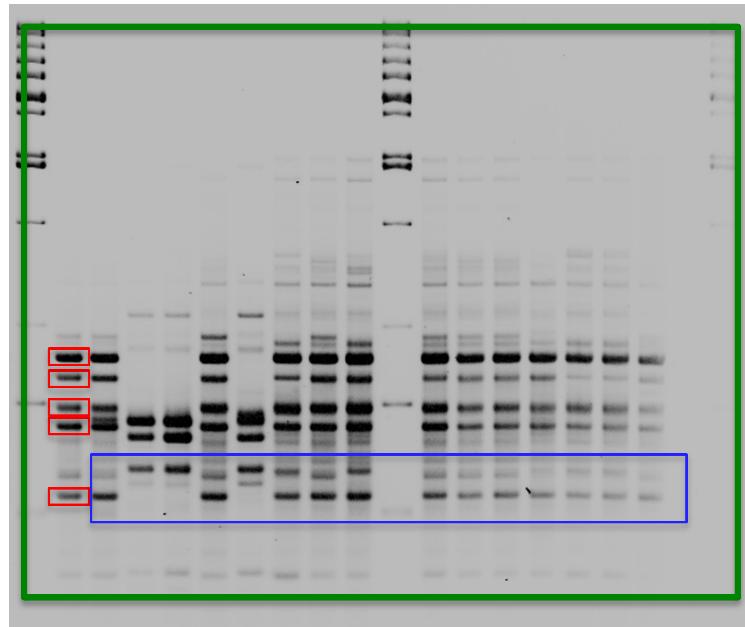
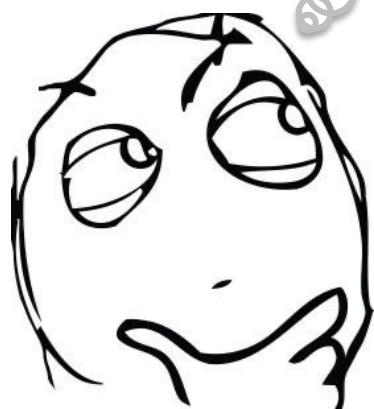
Strain level discrimination...



The method has strain level discrimination: (we are looking at the same or different strains)

But the gel does not tell us anything about taxonomic classification... (we do not know what we are looking at)

Could we sequence it ?



Sanger sequencing

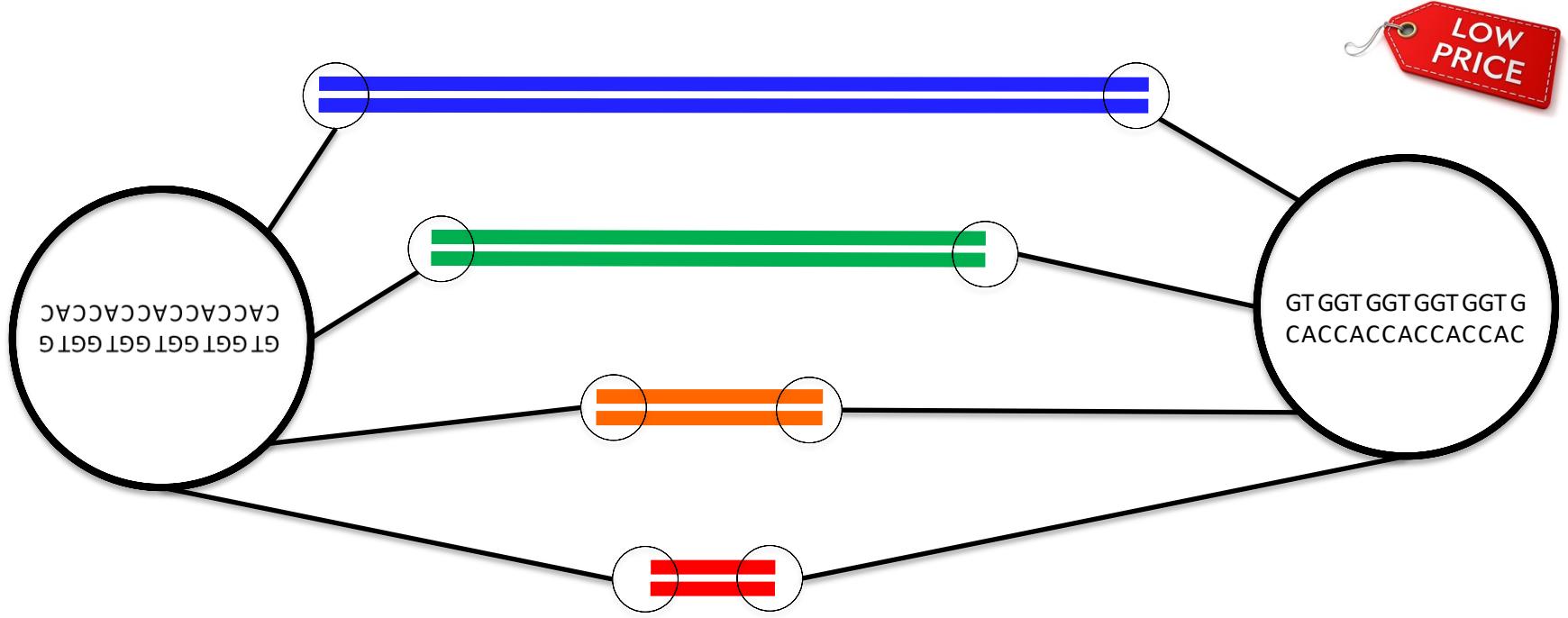
Can sequence DNA 100bp -1KB
But one band at a time...

Illumina based sequencing

can sequence mixed bands but only up to ~500bp

Oxford Nanopore Technology sequencing

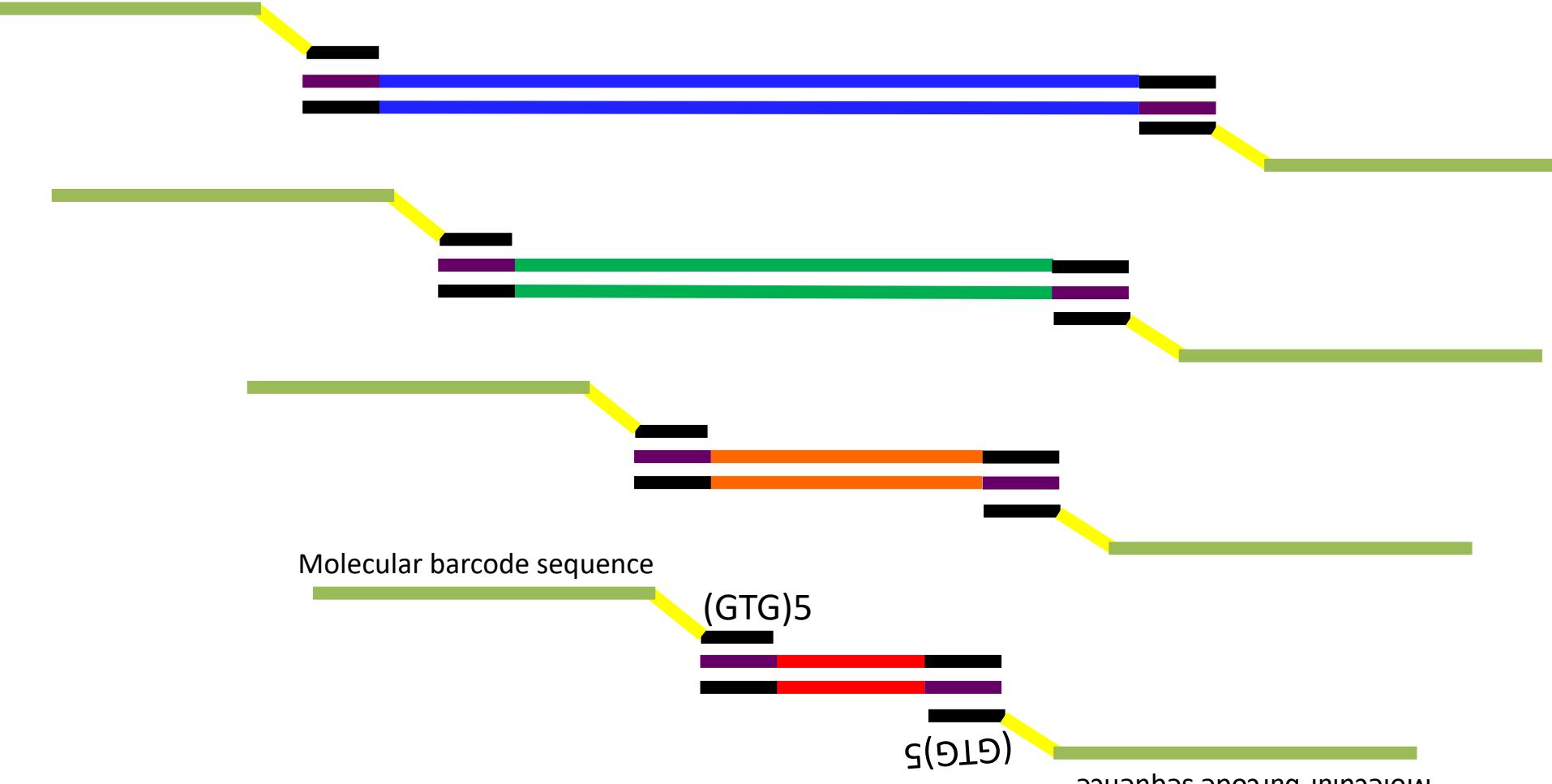
Could get it all...

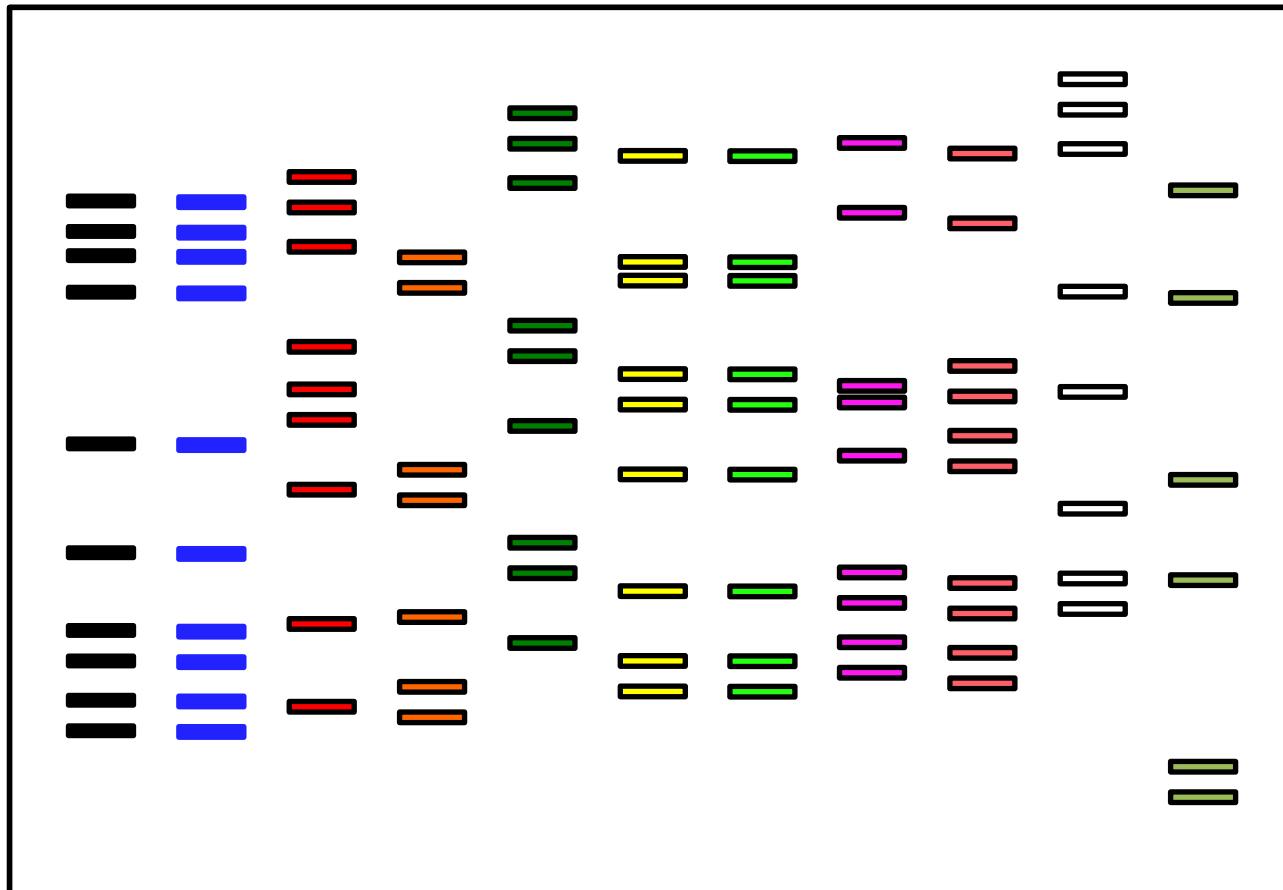


Each amplicon from rPCR begins and end with (GTG)5 sequence

Barcoding

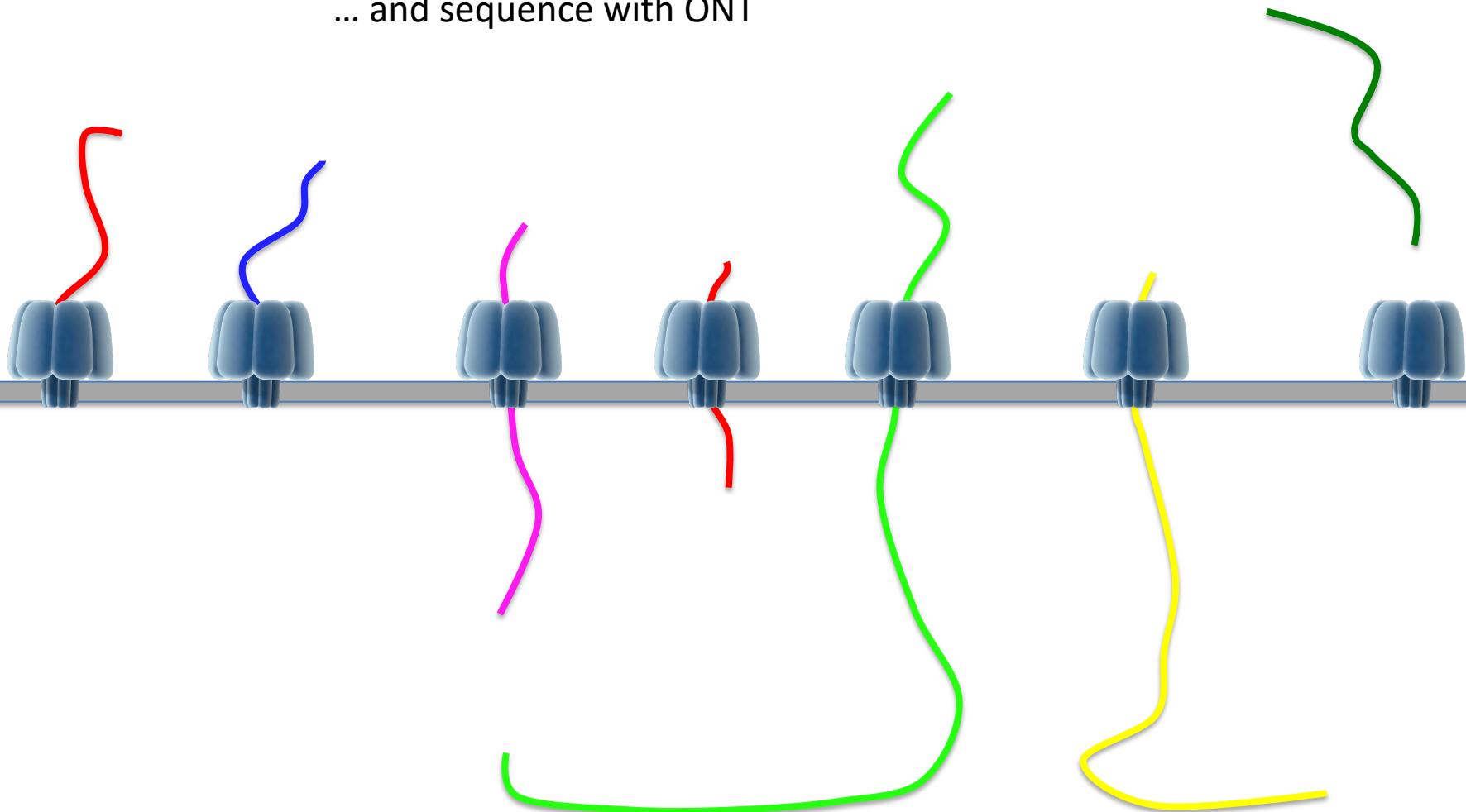
LC
2018
LONDON
CALLING



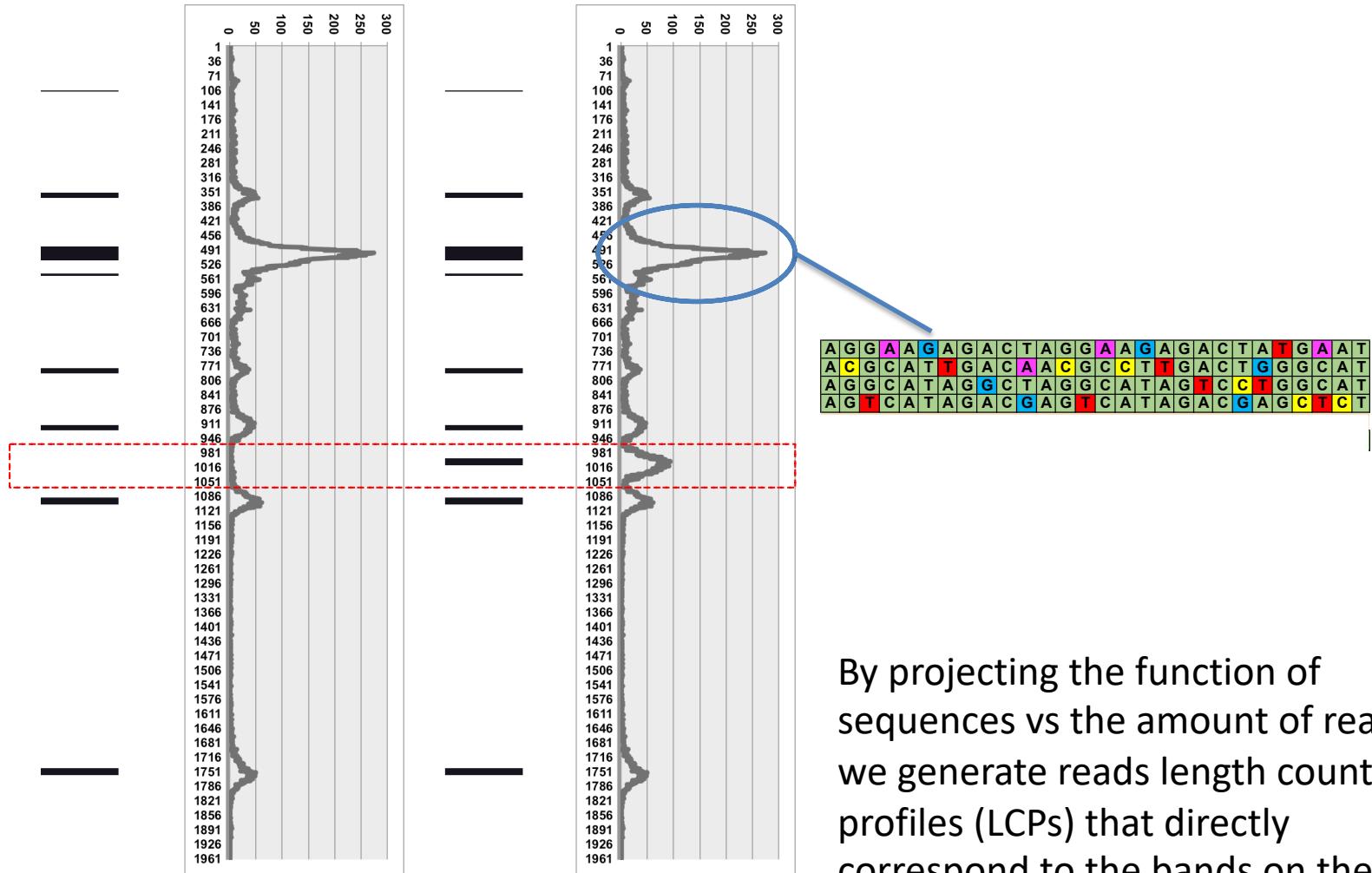


Once barcoded we can mix it all in one tube....

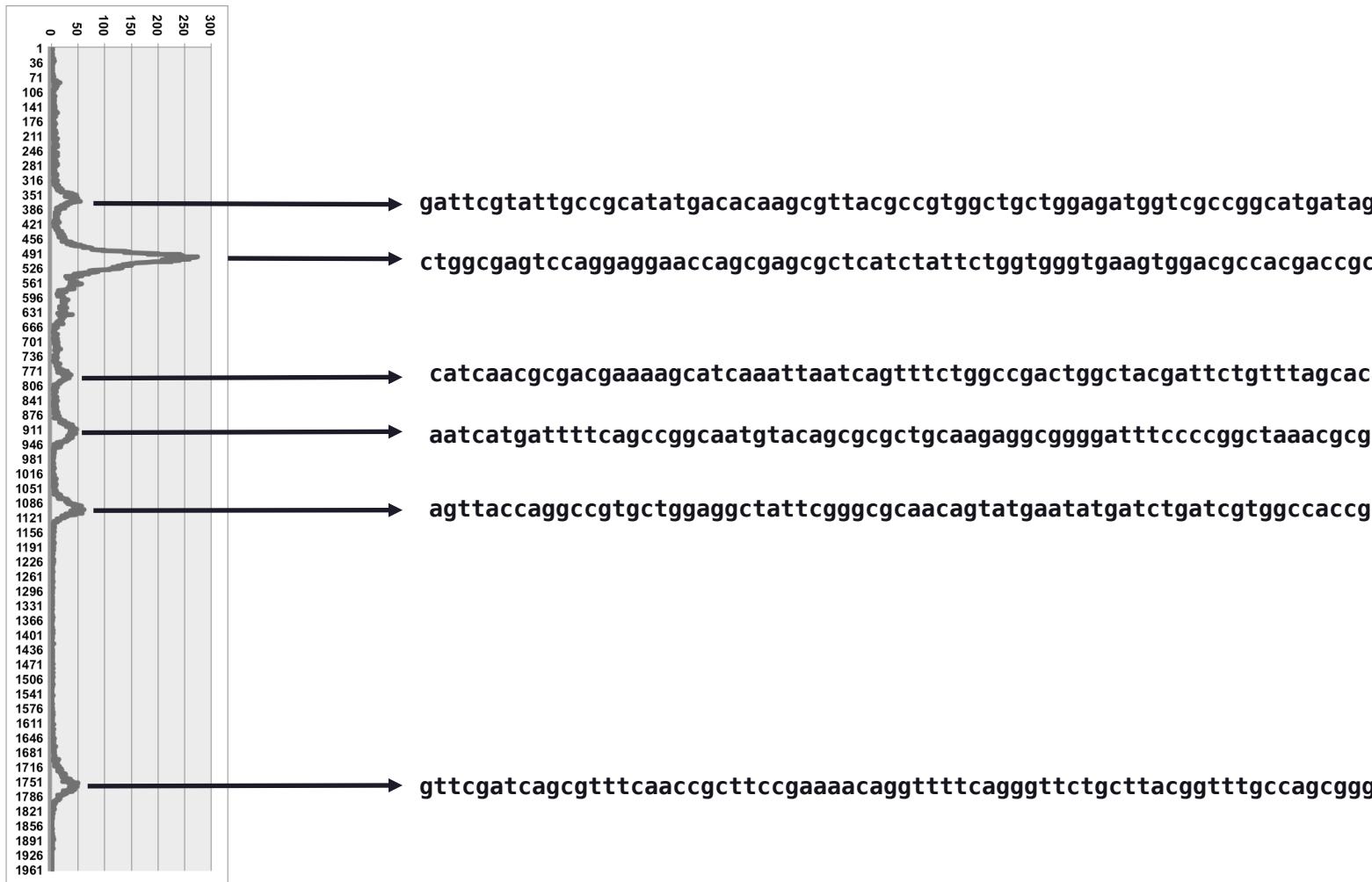
... and sequence with ONT



ON-rep-seq



ON-rep-seq



Only this time, not only we have the LCPs (bands on gel) but also the sequence, therefore we can ask database for taxonomy

ON-rep-seq

Identity 92%

CTGATTCACACCGCAGCCTCGCTGCCAGTGCCTGACCTAT-GCCGTCCCC-AAAG
CTGATTCACACCGCAGCCTCGCTGGC-GGTG-CTG-TTTGTAACCGTCCCCAAAG

ACGCTAACGAA--GGACCGCCGTGCCCCATTATGCTGATCCCACCGAAC
ACGCTAACGAAAGAGGATAACCGTGTCCGTATT-ATTATGCTGATCCCACCGAAC

AGCCCTGGCCTGACCACCGTCAGCCCTGGCGTCATC-CTGCTATGGAGACCCAAGGGT
AGCCTGGCCTGACCACCGTCAGCCCTGGCGTCATCCGTCTATGGA-ACGCAAAGGGT

T-GTCTGATGC-AC--AAGCTATGCCCAAGCCTCGCGCTGGCGAGT-CG-C-GACC
TCGCTGA-GCCCTTTAACGCTATGCCCAAGCCTGGCGCTGGCGGATGCCCTGACC

AGACCAACCAACTATGTTGCCGGAACTCTACCCCTGCCGGCGCTGAACCGCTGAAGATGA
AGACCAACCAACTATGTTGCCGGAACTCTACCCCTGCCGGCGCTGAACCGCTGAAGATGA

GCCACGTTGAATCTCTGCTCTCCAGCAACCAAGAAAAGACGTGCTGATGGAA-AGATCATC
GCCACGTTGAATCTCTGCTCTCCAGCAACCAAGAAA-GACGTGCTGATGGAAAGAGATCAT-

CGCGAACTACCATGCGAATACCAAAAGACCGGGAAAGTGGTGGCTGGTGAAGGCTGGTCC
CGCGAACTACCATGCGAATACCAAAAGACCGGGAAAGTGGTGGCTGGTGAAGGCTGGTCC

GGCCCGTAAACATCAGTCGCTCAGTCGCTGAACTATGAAATCGCGAAAACGCTGAATGC
GACCGTAAACATCAGTCGCTCAGTCGCTGAACTATGAAATCGCGAAAACGCTGAATGC

Identity 99%

TTCCCAAGAAATGATGCGTTGTGCGAAACCAATTTCGAATTGCGCCGCTGCTGCCG
TTCCCAAGAAATGATGCGTTGTGCGAAACCAATTTCGAATTGCGCCGCTGCTGCCG

CGCAATGAGC-CCCGGGTGAACGGTGAAGCTATCAGGTGGCAACGTACAATATCGGTTA
CGCAATGACGCCCGGTGAACGGTGAAGCTATCAGGTGGCAACGTACAATATCGGTTA

ACGATTGTTGAATCGACCCGATACACTACGCTTGTAACGATCGAGCAAACGACGCCAGCC
ACGATTGTTGAATCGACCCGATACACTACGCTTGTAACGATCGAGCAAACGACGCCAGCC

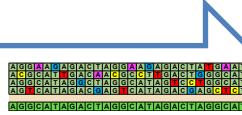
ATCACCTACTGGACCCCTCCGCTGCGTACGGTGGCCCTACCATGATGGATGGTGGCT
ATCACCTACTGGACCCCTCCGCTGCGTACGGTGGCCCTACCATGATGGATGGTGGCT

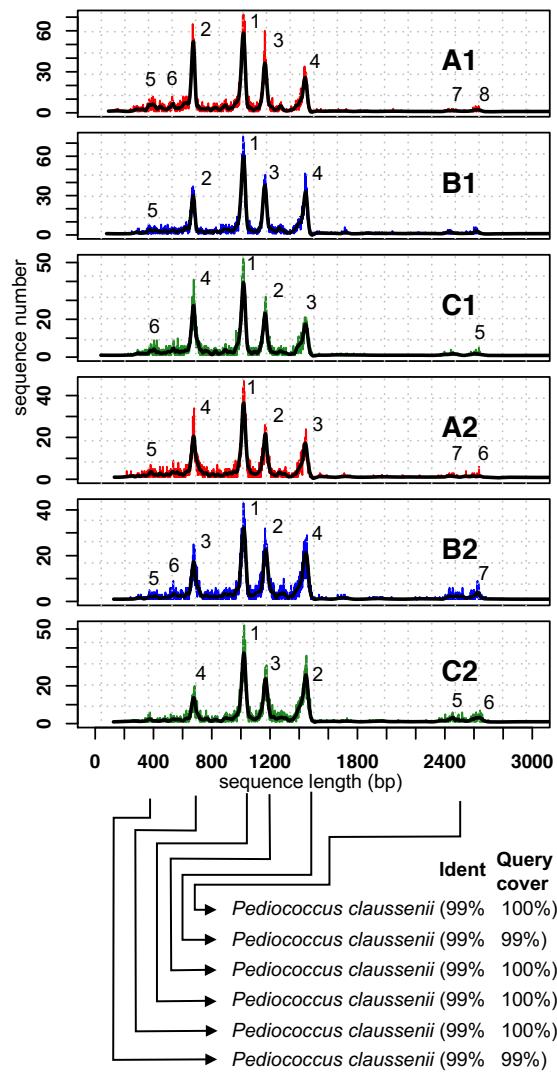
GAAGTGTGTTCAAGCCACGAGATCTTCGCTTCAAGCCGGTATGTTATCAAAT-AA
GAAGTGTGTTCAAGCCACGAGATCTTCGCTTCAAGCCGGTATGTTATCAAATAAA

AAGTTGCATCAACCGGACGAAAAGCATCAAAATTAAATCAGTTCTGGCGACTGGCTACGA
AAGTTGCATCAACCGGACGAAAAGCATCAAAATTAAATCAGTTCTGGCGACTGGCTACGA

TTCTGTTAGCACATGGAGCGATGGCGATTCCGGTTTATTAGCGTCGTGAAACCTAAGGA
TTCTGTTAGCACATGGAGCGATGGCGATTCCGGTTTATTAGCGTCGTGAAACCTAAGGA

CACCATTTGAAAGCCCTGTTAACCTACCTGGCTGGTGAAGGCGAGTCAGGATTTTA
CACCATTTGAAAGCCCTGTTAACCTACCTGGCTGGTGAAGGCGAGTCAGGATTTTA





Tested throughput: 384 isolates per flow cell

Theoretical throughput up to 1200 isolates per single flow cell

4h run A (0.94 M reads)

↓ washing / storing 1 day

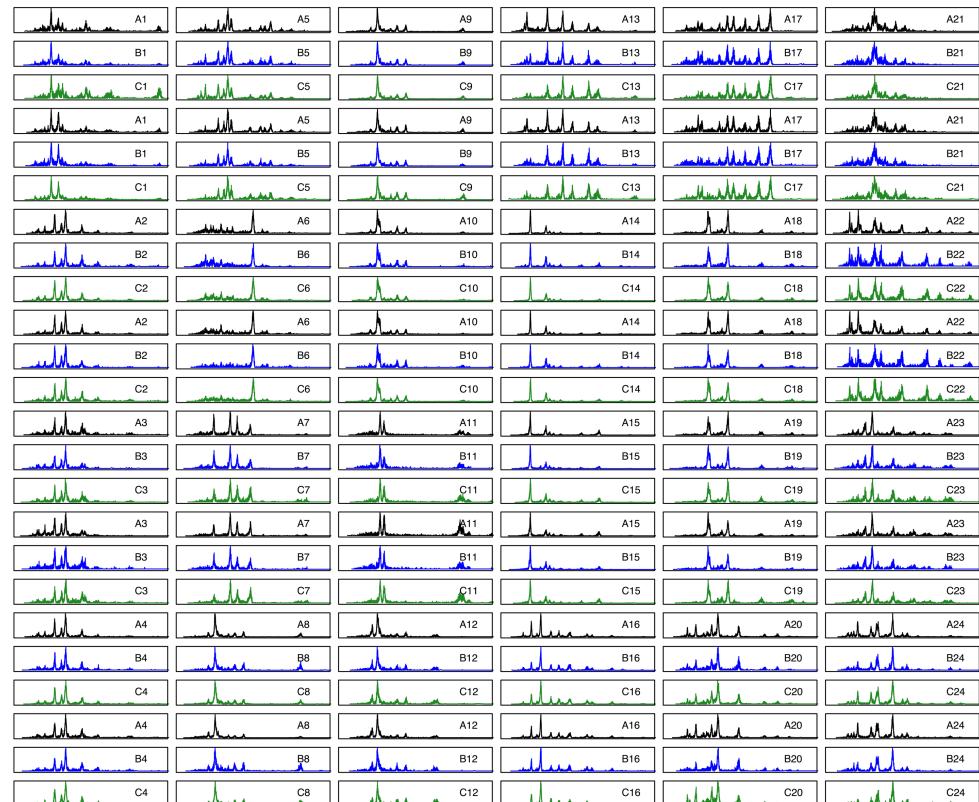
4h run B (0.79 M reads)

↓ washing / storing 4 days

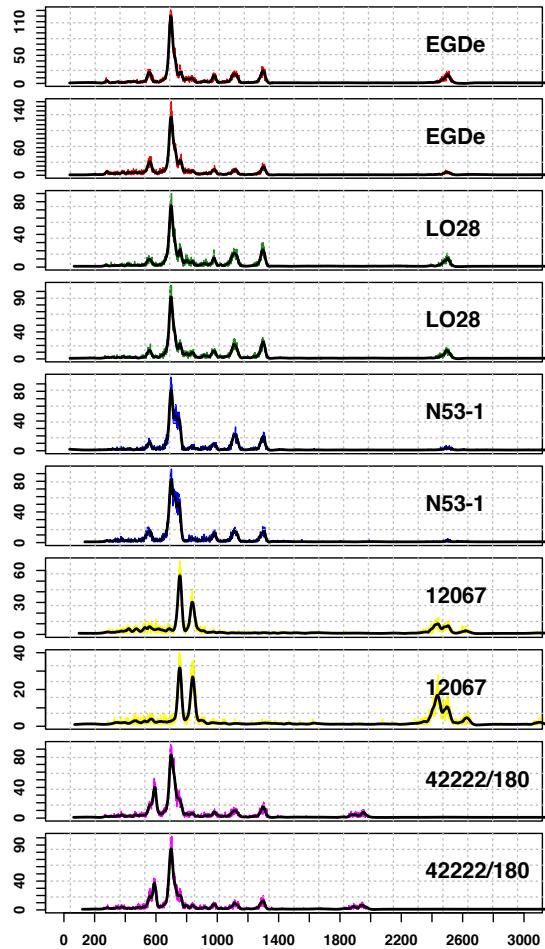
4h run C (0.57 M reads)

↓ washing / storing 3 days

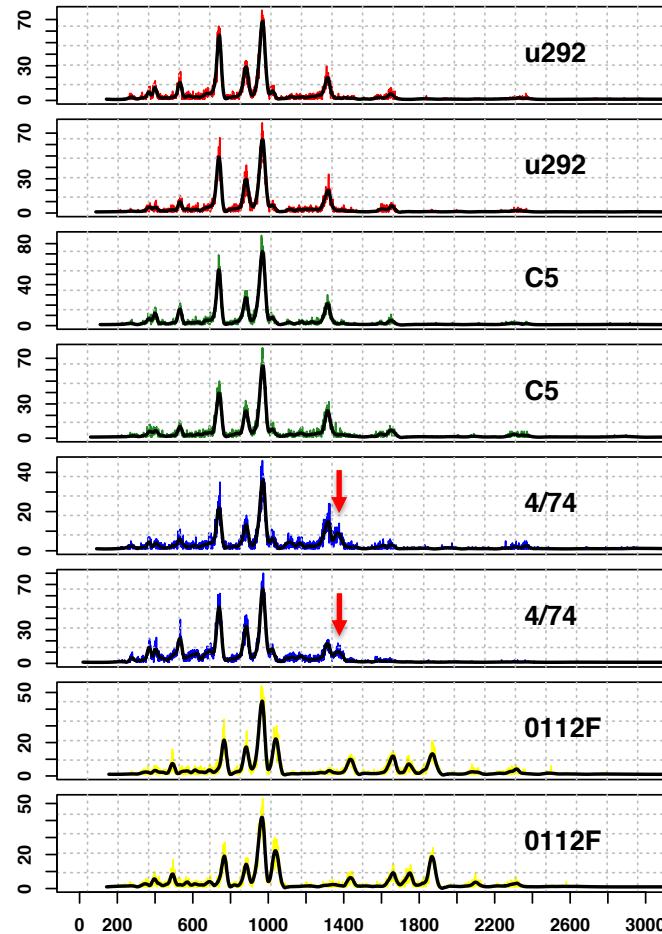
4h run D (0.22 M reads)



Listeria monocytogenes

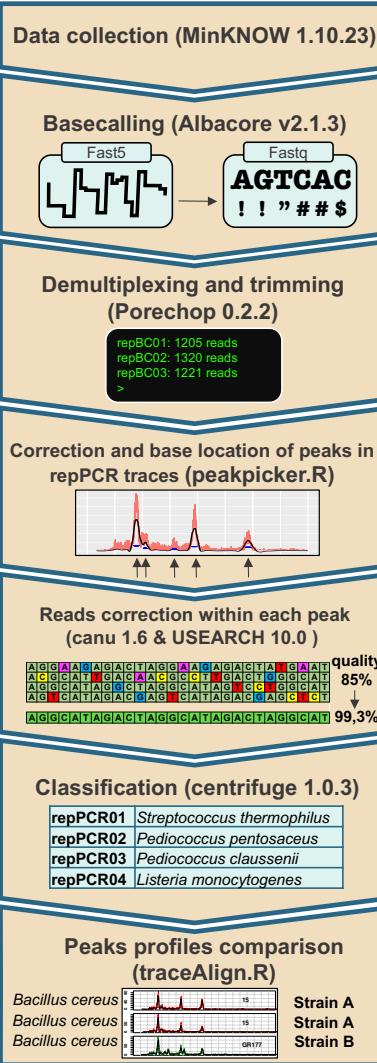
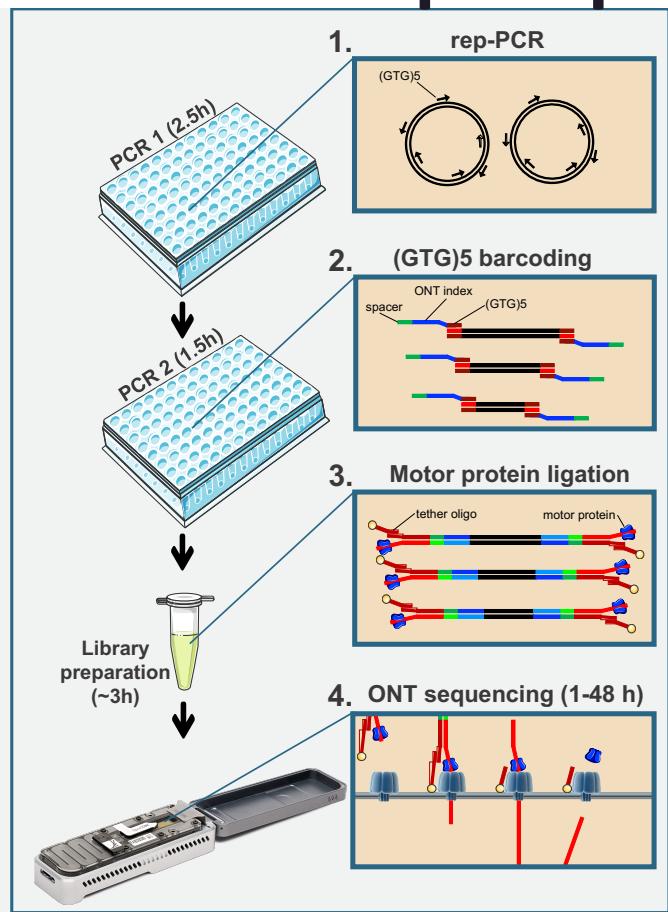


Salmonella enterica



Pipeline

• ON-rep-seq



?



Yeasts in Fermented Food and Beverages

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UNIVERSITY OF COPENHAGEN



Intended leaning outcome:

- ✓ You should know the predominant yeast species relevant for the fermentation of important food and beverages
- ✓ You should have knowledge on the major role of yeasts in fermented foods based on examples on technological properties as e.g. aroma and alcohol production, inhibition of pathogens, degradation of toxins, synthesis of micronutrients etc.
- ✓ You should be able to reflected on aspects related to the functionality of yeasts in food and beverages, e.g. interactions with other microorganisms, ability to adapt to environmental changes, importance for product quality and human health

Background: Yeasts are important for the quality of many foods and beverages



Origins of yeasts

- Spontaneous fermentation

Raw materials

Process equipment

- Inoculum

Part of previous fermentation (back-slopping)

Pre-fermentation

- Starter cultures

Dried yeasts

Direct inoculation



Yeasts in fermented products

- Brewing yeasts
- Winery yeasts
- Sourdough
- Yeasts used for production of cheeses
- Yeasts used for meat products
- Indigenous fermented products
- Yeasts in cash crops (cocoa, coffee etc.)
- Probiotic yeasts



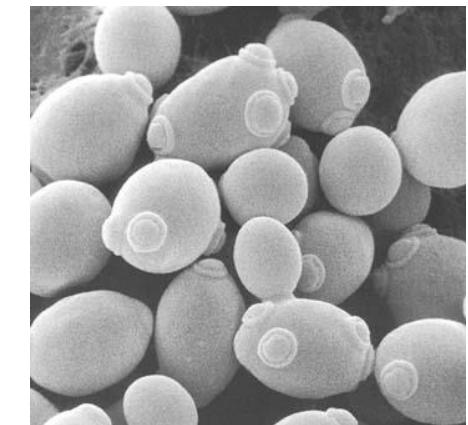
5 min discussion with your neighbours (2-3 persons)

- Choose two types of yeast fermented products mentioned on the previous slide.
- Suggest whether the yeasts occur i) spontaneously, ii) are added as single strains, iii) mixed cultures of species/strains or iv) the fermentation is a result of both added starter cultures and spontaneous fermentation.
- Describe for the two products the main functionality of the yeasts
- Prepare for plenum discussion.



Functions of yeasts in fermented products

- Fermentation of carbohydrates (formation of alcohols etc)
- Production of aroma compounds (esters, alcohols, organic acids, carbonyls)
- Stimulation of LAB (pyruvate, amino acids and vitamins)
- Inhibition of mycotoxin producing moulds (nutrient competition, production of toxic compounds, killer toxins a.o.)
- Degradation of cyanogenic glycosides (linamarase activity)
- Production of tissue degrading enzymes (cellulases and pectinases)
- Production of glucoamylases
- Phytase activity
- Production of amino acids, higher protein efficiency (yeast-LAB)
- Probiotic effects
- Production of killer toxins
- Inhibition of pathogens



Brewing yeasts

Lager yeast (bottom yeast)

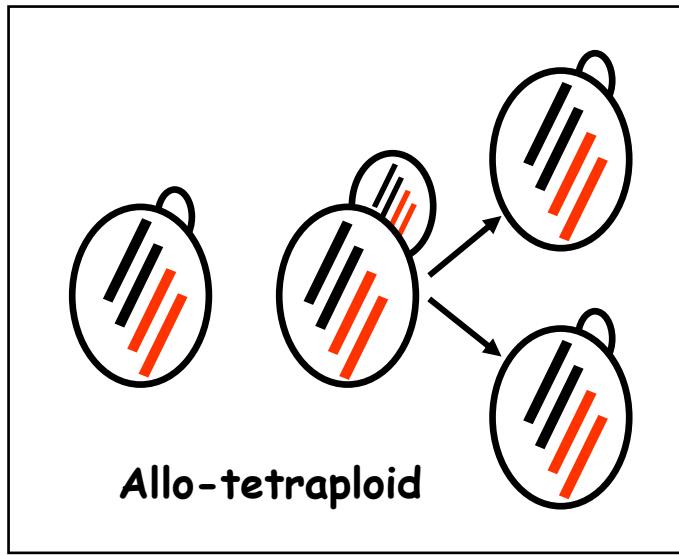
Species:	<i>Saccharomyces pastorianus</i> *
	(previous names: <i>S. uvarum</i> , <i>S. carlsbergensis</i> , <i>S. cerevisiae</i>)
Melibiase:	Positive
Growth at 37°C:	Negative
Production:	Lager beer

Ale yeast (top yeast)

Species:	<i>Saccharomyces cerevisiae</i>
Melibiase:	Negative
Growth at 37°C:	Positive
Production:	Ale, Stout and some others

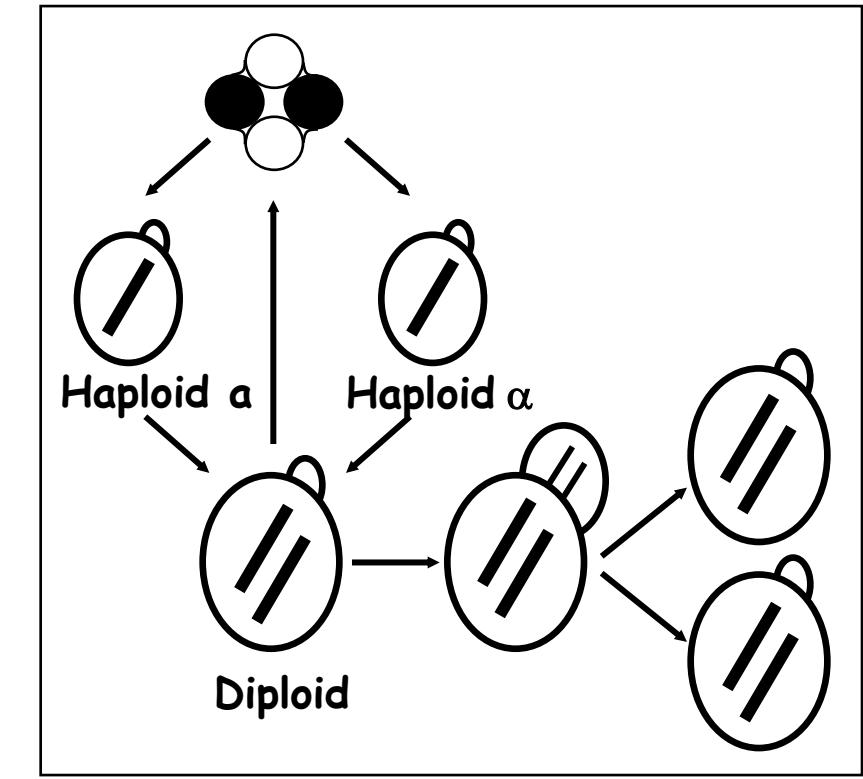
* according to Kurtzman and Fell (1998)

Chromosomal arrangement in brewing yeasts



Chromosomal arrangement in *S. pastorianus*

Lager brewing strains are thought to originate from a natural hybridization event that occurred between an *S. cerevisiae* strain and a non-*S. cerevisiae* strain, probably an *S. eubayanus* strain



Chromosomal arrangement in *S. cerevisiae*

Mating type is determined by a single locus, *MAT*; **a** or **α** (alpha)

Technological characters of brewing yeasts

- Flocculence (high, moderate, weak, non-flocculent)
- Reproduction (adequate, inadequate)
- Attenuation (high, moderate, weak)
- Aromatic properties (aromatic, slightly aromatic, non-aromatic)
- Off flavours (clean, unclean; sulphury, diacetyl)



Aroma components in beer

AROMA COMPONENTS FORMED BY YEASTS

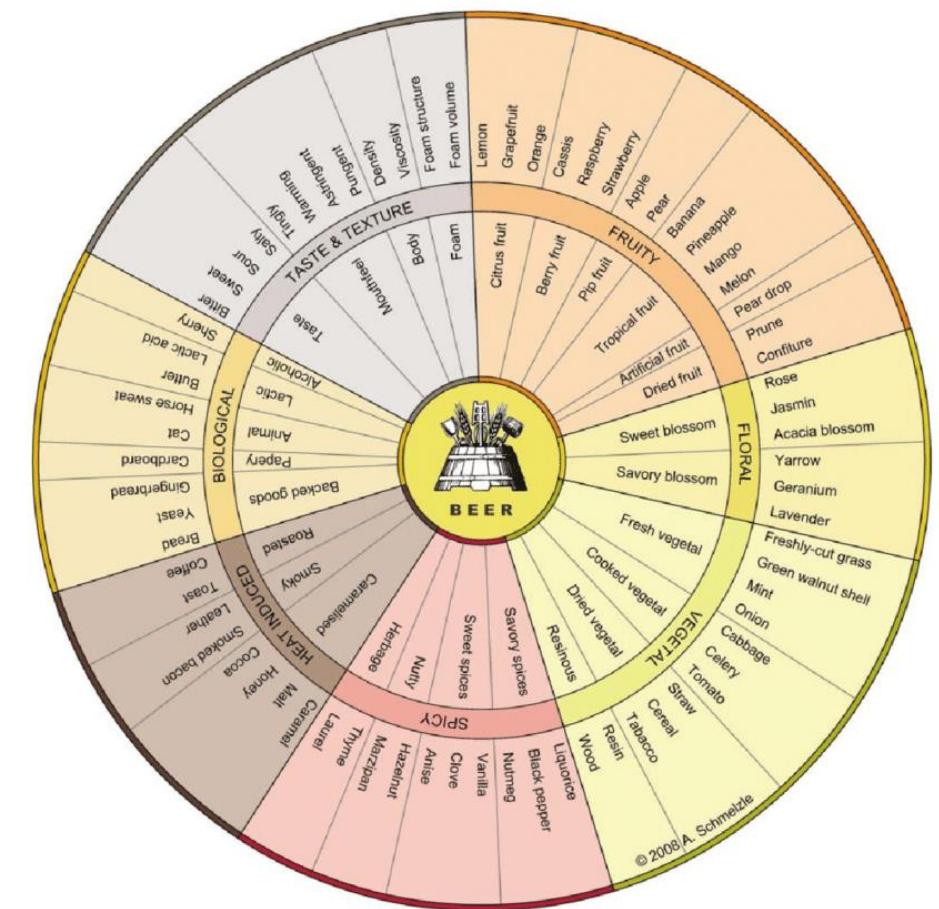
- Esters
 - Alcohols (fusels)
 - Vicinal diketones (diacetyl, 2,3-pentandion)
 - Aldehydes (acetaldehyde)
 - Organic acids
 - Sulphury compounds
 - Fatty acids

RAW MATERIALS

(malt, hops (isohumulon a.o)

OFF-FLAVOURS

(trans-2-nonenal, 3-methyl-2-buten-1-thiol, phenols, DMS a.o.)



Wine fermentation

Natural fermentation

Yeasts resident in the grape juice initiate and complete the fermentation

Varied outcome with the potential of failure but with the prospect of wines with more interesting character

Pure culture fermentation

Selected strains of *S. cerevisiae* are inoculated into the juice at initial populations of 10^6 to 10^7 cells/ml

More rapid and predictable fermentation

... and sometimes with non-Saccharomyces yeasts added

Winery Yeasts

Original flora

Surface of grapes:

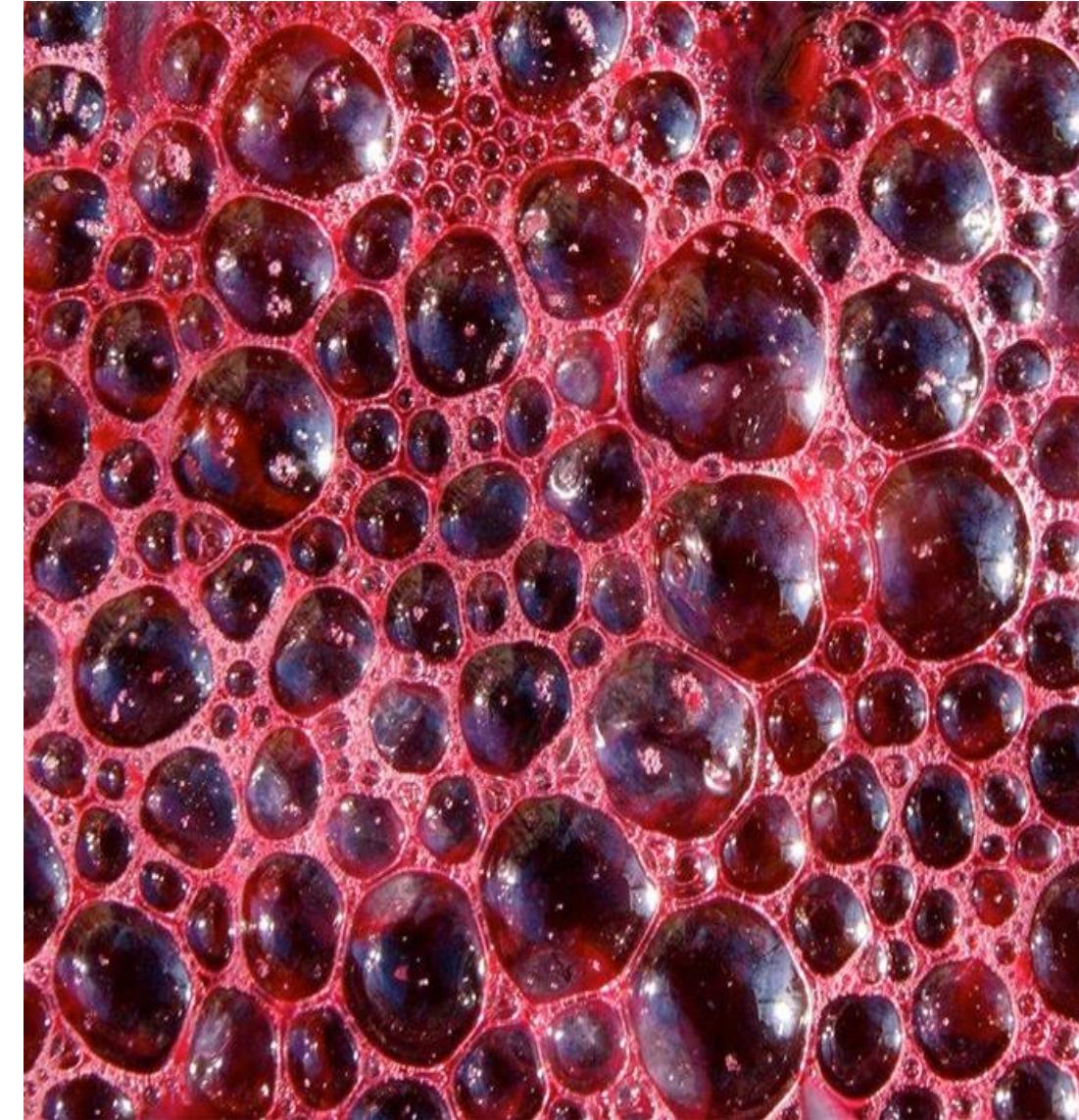
Kloeckera spp., *Hanseniaspora* spp.,
Kluyveromyces spp., *Metschnikowia* spp.,
Cryptococcus spp., *Pichia* spp., *Hansenula*
spp., (*Saccharomyces* spp.)

Surface of winery equipment/grapes

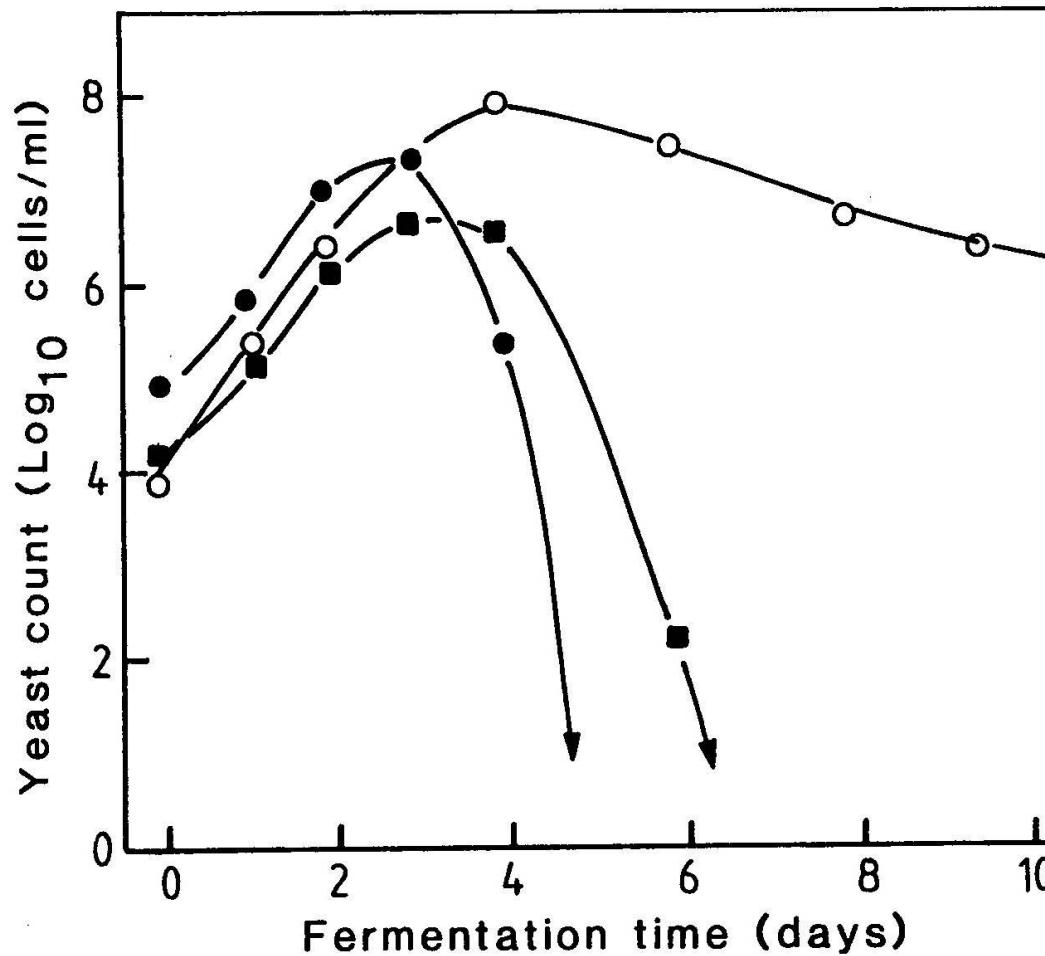
Saccharomyces cerevisiae

Starter cultures

Saccharomyces cerevisiae and non-
Saccharomyces yeasts as e.g. *Pichia kluyveri*



Wine fermentation - Succession of yeasts



White circles: *S. cerevisiae*
Black symbols: other yeasts



Sugar catabolism

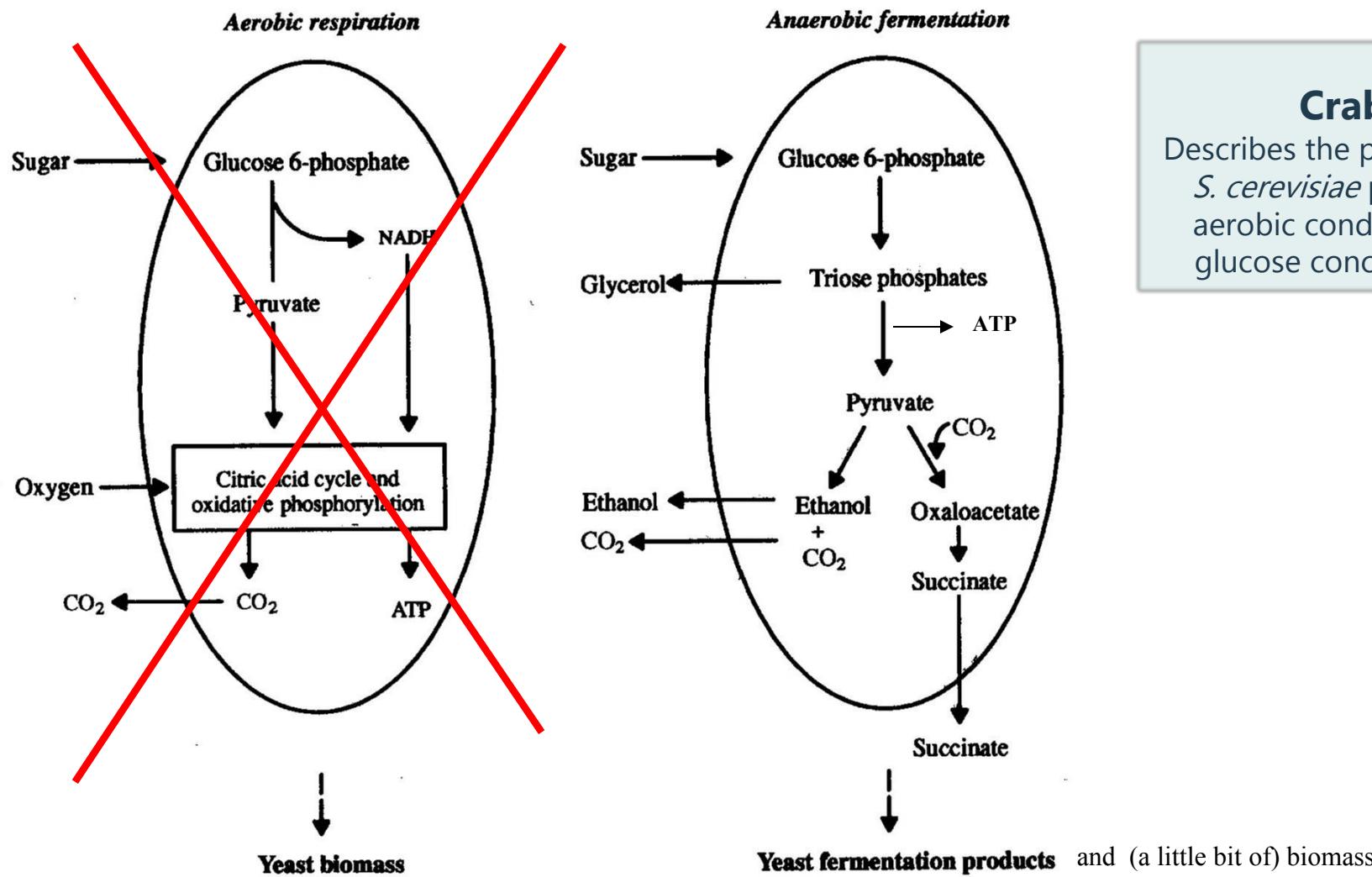


Figure 5.9. Summary of major sugar catabolic pathways in yeast cells.

Crabtree effect:

Describes the phenomenon whereby e.g. *S. cerevisiae* produces ethanol under aerobic conditions and high external glucose concentrations (> 150 mg/l)

Properties used to select yeasts for wine production

Desirable properties

High ethanol tolerance

High osmotolerance

Tolerance to low pH

Tolerance to anoxic conditions

Complete and rapid fermentation of sugars

Resistance to sulfur dioxide

Production of good flavour and aroma



Undesirable properties

Production of hydrogen sulfide

Production of volatile acidity

Inhibition of malolactic fermentation

Production of polyphenol oxidase (affects wine colour)

Formation of ethyl carbamate precursors

Production of any other undesired flavour and aroma

Yeasts used for baking

Baker's yeast: *Saccharomyces cerevisiae*



Other yeasts (sourdough):

Kazachstania exigua formerly *Saccharomyces exiguum* (*Candida holmi*)

Torulaspora delbrueckii (*Candida colliculosa*)

Meyerozyma guilliermondii (*Candida guilliermondii*)

Candida milleri

Pichia kudriavzevii (*Candida krusei*)

etc.

Baker's yeasts (*Saccharomyces cerevisiae*)

Technological properties

- Efficient fermentation of dough carbohydrates (especially maltose)
- Formation of CO₂
- Aroma formation (organic acids, aldehydes, ethanol, higher alcohols, esters and ketones)
- Effect on dough structure (glutathione and cysteine)
- Osmotolerance
- Acid tolerance
- Freeze-thaw resistance



Sourdough

- Yeasts and LAB - a stable ecosystem!
- The variety and numbers of species in the dough are influenced by several endogenous and exogenous factors (flour, temperature, time of fermentation redox potential etc.)
- Dough acidification (mainly LAB), production of CO₂, leavening, aroma production
- Aroma formation is influenced both by the microbial interactions and by the intrinsic conditions in the dough



Yeasts used for production of cheeses

- *Debaryomyces hansenii*

Surface ripened cheeses (starter cultures)

Blue and white mould cheeses

- *Saccharomyces cerevisiae*

Gorgonzola (starter cultures)

Blue and white mould cheeses

Some surface ripened cheeses

- *Yarrowia lipolytica*

Found in various cheeses

But yeasts can also occur spontaneously from the brine, by "back-slopping", the dairy environment, ingredients etc.



Cheeses and the role of yeasts

Bacterial surface-ripened cheeses as Danbo or Limburger

- Promote bacterial surface-growth
- Aroma compound production

Blue-veined cheeses as roquefort, gorgonzola, stilton, Danish blue

- Opens texture by lactose fermentation
- Increase pH by lactate assimilation
- Aroma compound production
- Interactions with *Penicillium roqueforti*-culture

White-mould cheeses as camembert

- Lipase, esterase, peptidase activity
- Aroma compound production
- Interactions with *Penicillium camemberti*-culture

The role of *D. hansenii* during production of surface ripened cheeses

- Lowering the acidity of the cheese surface allowing the growth of bacteria
- Synthesis of substances that stimulate the growth of bacteria (e.g. pantothenic acid, niacin, riboflavin)
- Production of proteolytic enzymes (activity against casein and polypeptides from casein)
- Production of flavour components

Pro- and eukaryotes in Danish brines for cheese production

Microorganism	Sample							
	A1	A2	A3	B1	C1	C2	D1	D2
<i>Arthrobacter</i>						2.10		
<i>Chromohalobacter</i>	5.34	3.67	4.15	4.16	3.56	4.27	2.82	3.32
<i>Corynebacterium</i>		0.21						
<i>Enterobacter</i>								0.11
<i>Halomonas</i>			3.12				0.25	
<i>Idiomarina</i>					0.51			
<i>Kocuria</i>			0.02		0.35	1.15		0.79
<i>Kushneria</i>					0.10			
<i>Lactobacillus</i>		0.02			0.51	0.84		
<i>Lactococcus</i>	2.88	4.46	4.09	3.66	4.37	2.48	4.98	4.64
<i>Leuconostoc</i>	3.35							
<i>Marinilactibacillus</i>					2.82			
<i>Microbacterium</i>				4.14				
<i>Micrococcus</i>				0.00				
<i>Pediococcus</i>		0.45						
<i>Planococcus</i>					1.24	0.03		
<i>Psychrobacter</i>			1.03	6.21		2.26		2.94
<i>Salinivibrio</i>				0.00				
<i>Staphylococcus</i>	2.04	1.22	0.61		1.44	0.83	5.28	0.45
<i>Tetragenococcus</i>	3.95	4.31		4.91	0.27		5.42	4.45
<i>Candida</i>						0.77	0.07	
<i>Debaryomyces</i>	3.70	3.84	2.12		2.09	3.27		
<i>Kluyveromyces</i>			0.10					
<i>Papiliotrema</i>				0.66				
<i>Penicillium</i>						0.02		
<i>Rhodotorula</i>						0.02		
<i>Sterigmatomyces</i>					0.61	0.02		
<i>Yamadazyma</i>							1.03	2.47

Log CFU ml⁻¹. For microorganisms observed at multiple substrates, the overall highest level is listed.



Functions of yeasts (*Debaryomyces hansenii*) in meat products

Microenvironment in meat products:

- High concentration of protein and lipids
- Low temperature
- Low a_w (high NaCl concentrations)



Functions of yeasts in meat products:

- Aroma formation
- Proteolytic and lipolytic activity
- Depletion of oxygen (colour stabilisation)
- Reduction of nitrate
- Prevention of the growth of mycotoxin producing moulds



Debaryomyces hansenii for bioprotection and prevention of ochratoxin formation

Debaryomyces hansenii can act as a biopreservative agent to inhibit the growth of ochratoxigenic moulds and reduce OTA accumulation in dry cured meat

International Journal of Food Microbiology 170 (2014) 70–77



Inhibition of ochratoxigenic moulds by *Debaryomyces hansenii* strains for biopreservation of dry-cured meat products

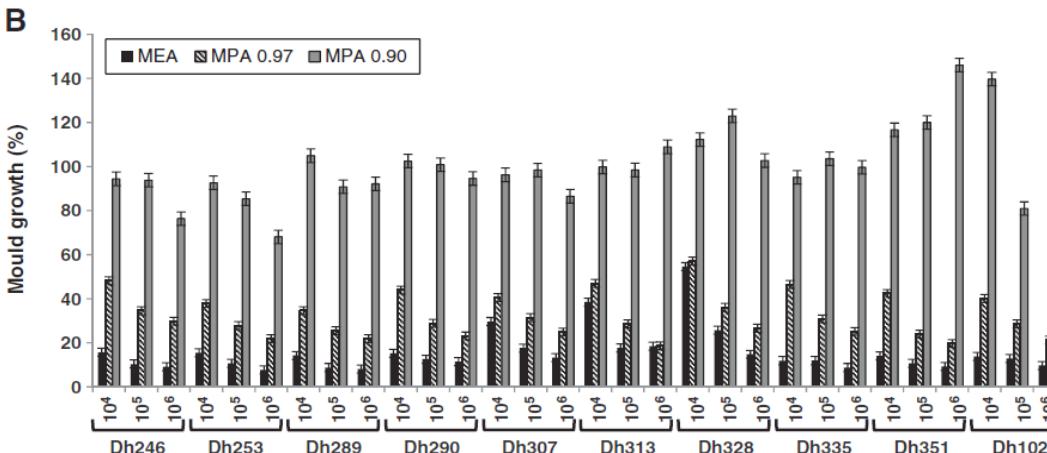
Maria J. Andrade ^{a,*}, Line Thorsen ^b, Alicia Rodríguez ^a, Juan J. Córdoba ^a, Lene Jespersen ^b

^a Food Hygiene and Safety, Faculty of Veterinary Medicine, University of Extremadura, Spain

^b Department of Food Science, Food Microbiology, Faculty of Science, University of Copenhagen, Denmark

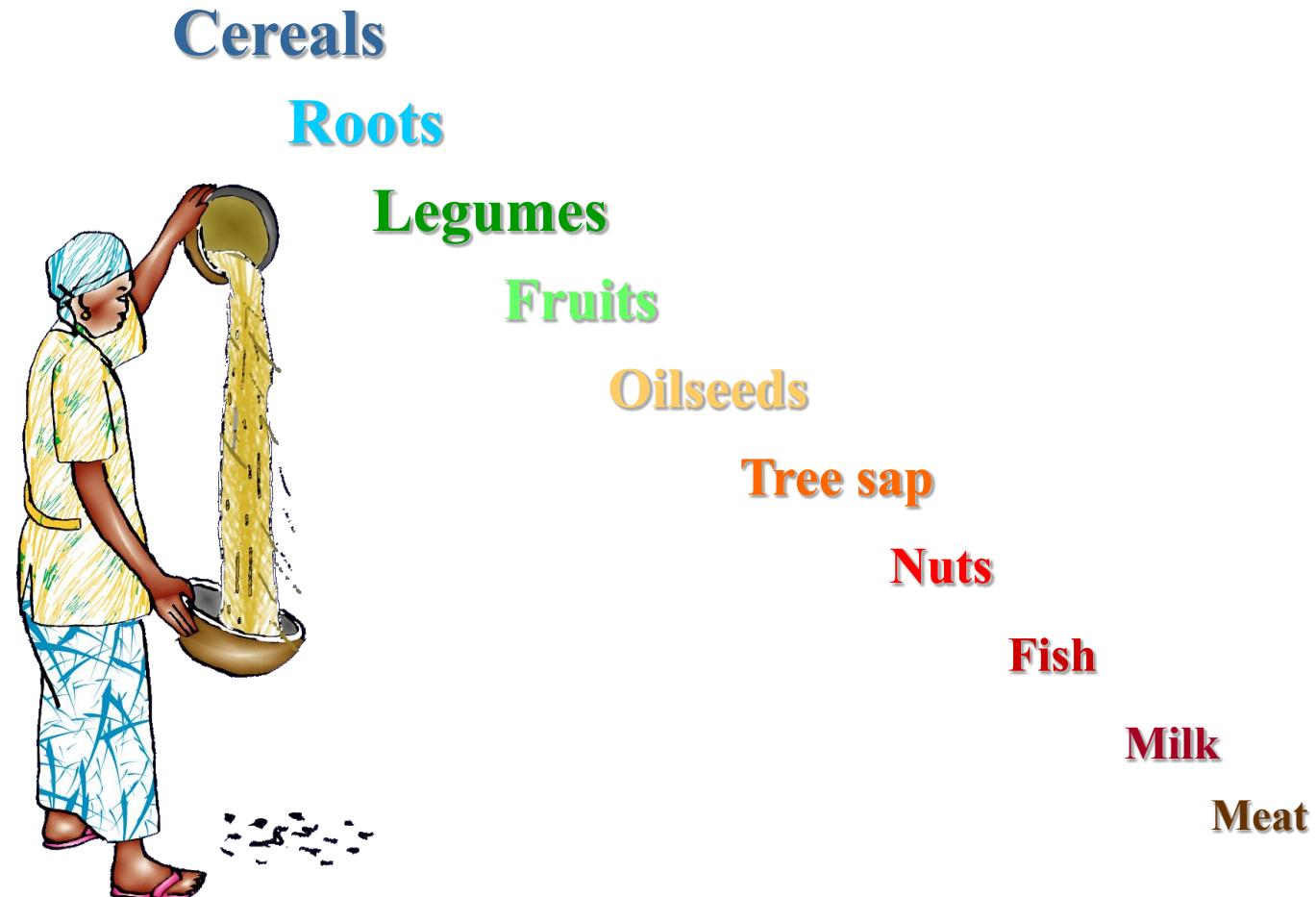


D. hansenii strains and inoculum level (cfu/mL)



	OTA production (µg/kg)	
	15 days	30 days
<i>P. nordicum</i>	42.9 ± 8.0	37.4 ± 10.4
<i>P. nordicum</i> + <i>D. hansenii</i>	26.7 ± 5.2	29.9 ± 5.7

Sources used for indigenous fermented food and beverages in Africa



Examples on co-existence between *S. cerevisiae* and other microorganisms in indigenous food and beverages

Alcoholic beverages

Sorghum beer: LAB, *Candida krusei*, *Candida* spp., *Kluyveromyces* spp., *Torulaspora delbrueckii*

Palm wine: LAB, acetic acid bacteria, *Candida krusei*, *Schizosaccharomyces pombe*, *Pichia* spp., *Candida* spp.

Maize beverage: LAB, *Candida krusei*

Non-alcoholic starchy foods (maize)

"Kenkey": LAB, *Candida krusei*

"Ogi": LAB, acetic acid bacteria, *Corynebacterium* spp., *Zygosaccharomyces rouxii*, *Candida vini*

"Togwa": LAB, *Candida krusei*, *Wicherhamomyces anomalus*, *Candida tropicalis*

Fermented milk

"Rob": LAB, *Kluyveromyces marxianus*

"Amasi": LAB, *Torulaspora delbrueckii*, *Naumovozyma dairenensis*, *Clavispora lusitaniae*

Yeasts and human health

Phytase activity of yeasts – huge strain variations

Table 4 Volumetric (mU ml^{-1}) and specific extracellular [$\text{mU (10}^{10} \text{ CFU)}^{-1}$] and intracellular (mU mg^{-1} total protein) phytase activities from yeast strains of *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Candida krusei* and *Arxula adeninivorans*

Species	Strains	Phytase activities*			
		Extracellular		Intracellular	
		mU ml^{-1}	$\text{mU (10}^{10} \text{ CFU)}^{-1}$	mU ml^{-1}	mU mg^{-1} total protein
<i>S. cerevisiae</i>	ATCC26108	9 ± 1	281 ± 22	6 ± 1	4 ± 1
	DGI342	10 ± 1	62 ± 7	6 ± 1	3 ± 1
	CBS1236	3 ± 1	28 ± 2	20 ± 3	12 ± 1
	Pito	28 ± 1	457 ± 52	14 ± 2	8 ± 1
	KVL013	3 ± 1	83 ± 28	30 ± 5	17 ± 3
	KVL015	67 ± 1	732 ± 8	36 ± 1	22 ± 3
<i>S. pastorianus</i>	KVL008	76 ± 6	1981 ± 20	14 ± 2	8 ± 1
	KVL016	3 ± 1	26 ± 3	19 ± 3	11 ± 0
<i>C. krusei</i>	Kenkey	30 ± 5	509 ± 62	18 ± 1	11 ± 2
	Kenkey	20 ± 3	111 ± 18	17 ± 2	10 ± 2
	Pito	14 ± 2	110 ± 5	46 ± 2	27 ± 3
	Pito	50 ± 14	595 ± 58	19 ± 5	11 ± 2
	Pito	35 ± 3	460 ± 53	15 ± 1	9 ± 2
	<i>A. adeninivorans</i>	CBS7377	61 ± 5	519 ± 38	6 ± 1

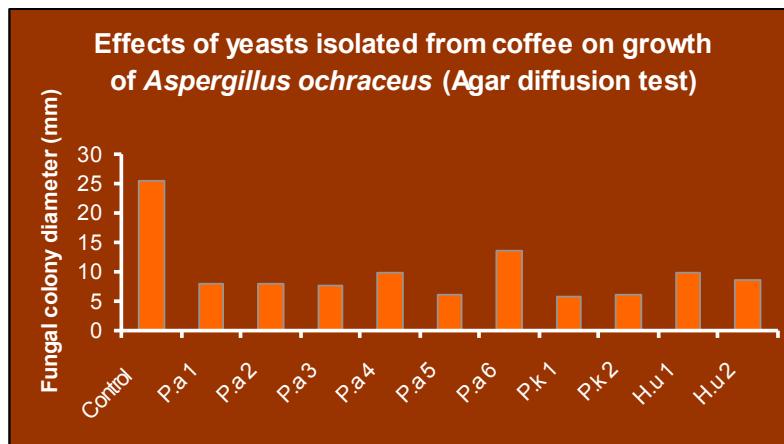
*Results are expressed as the mean of three replicated measurements and standard error of the mean ($\pm\text{SEM}$).

Yeast – coffee fermentation

Processing of coffee beans in East Africa



Predominant yeasts in coffee can inhibit ochratoxin producing moulds



P.a: *Pichia anomala* (now *Wicherhamomyces anomalus*),
P.k: *Pichia kluyveri*, H.u: *Hanseniaspora uvarum*

Masoud and Jespersen 2006 Int. J. Food Microbiol. 110, 291-296

Pectinolytic activity of yeast species in coffee fermentation

Yeast species	Growth on pectin agar
<i>Candida pseudointermedia</i>	++
<i>Wicherhamomyces anomalus</i>	++
<i>Pichia kluyveri</i>	++
<i>Kodamaea ohmeri</i>	-
<i>Candida krusei</i>	-
<i>Kluyveromyces marxianus</i>	+
<i>Hanseniaspora uvarum</i>	-
<i>Torulaspora delbrueckii</i>	-

++: good growth; +: weak growth; -: no growth

In conclusion...

- ✓ Several yeasts species are of major importance for many of our daily food and beverages
- ✓ Yeasts offer a variety of different technological properties (aroma and alcohol production, inhibition of pathogens, degradation of toxins, synthesis of micronutrients etc.)
- ✓ Yeast are used as starter cultures or take part of a spontaneous fermentation. Several yeast species may occur leading to microbial successions taking place at both species and sub-species level
- ✓ Yeast might have beneficial health effects and can enhance the bioavailability of micronutrients and produce vitamins



When microorganisms talk (Quorum Sensing)

Lene Jespersen, Professor

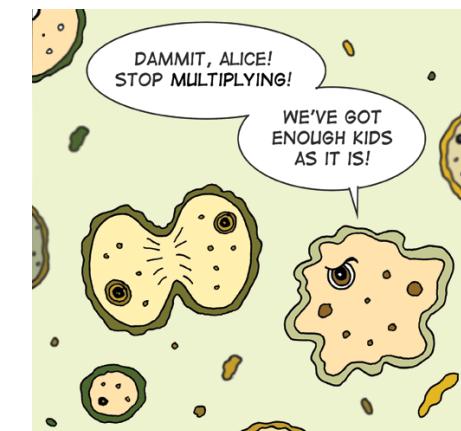
Department of Food Science, Food Microbiology
University of Copenhagen
lj@food.ku.dk

UNIVERSITY OF COPENHAGEN



Intended leaning outcome:

- ✓ You should have knowledge on different sensing mechanisms in bacteria and yeasts
- ✓ As an example, you should be able to describe quorum sensing (QS) in specific details
- ✓ You should be able to elaborate on, how and when QS could be a relevant parameter to consider during fermentation of food and beverages



Quorum Sensing – background and definition

- Quorum sensing is the regulation of gene expression in response to cell-population density
- Quorum sensing microorganisms produce and release chemical signal molecules that increase in concentration as a function of cell density
- Detection of a minimal threshold stimulatory concentration of a signal molecule leads to an alteration in gene expression
- Regulatory functions include many different microbial traits. The most well described are virulence expression and biofilm formation
- Quorum sensing allows unicellular micro-organisms to act as multicellular organisms

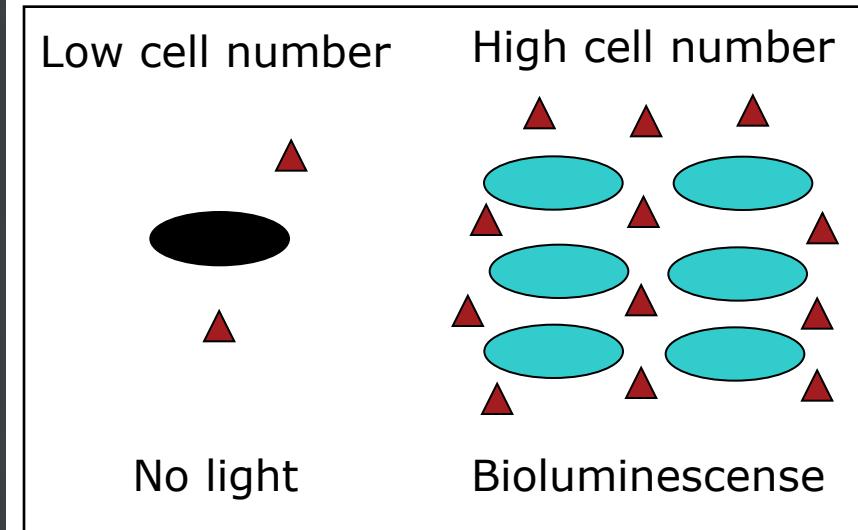


Social behaviours of microorganisms

Quorum sensing is the process by which microorganisms communicate by signalling molecules and change gene expression/behaviour in a cell density dependent manner



The marine bacteria
Vibrio fischeri

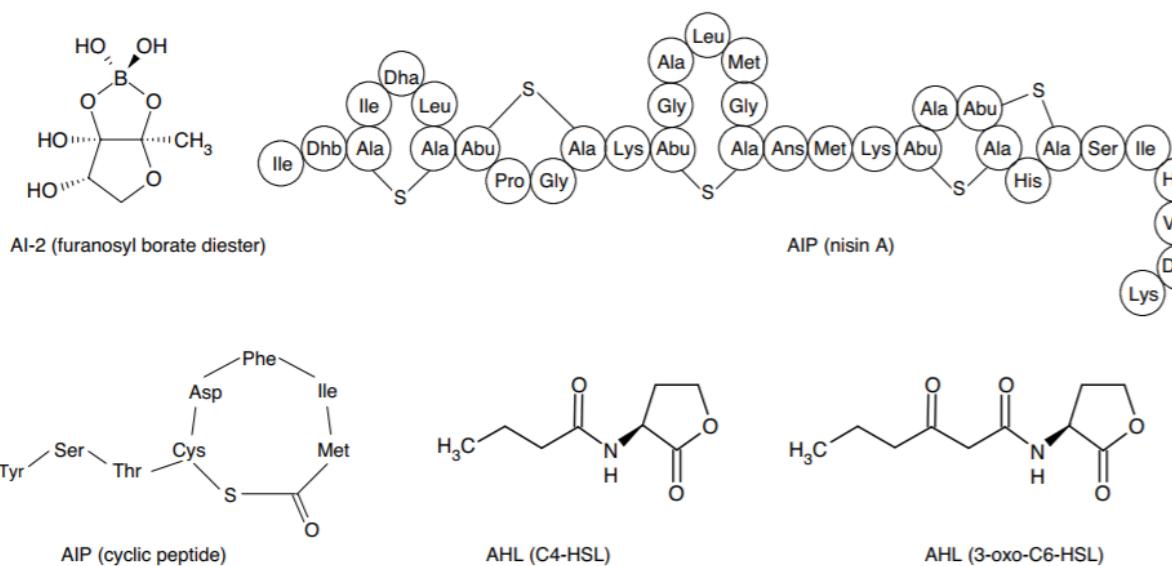


The Hawaiian bobtail squid

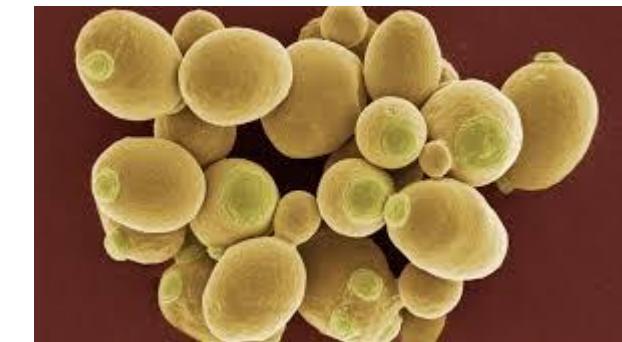
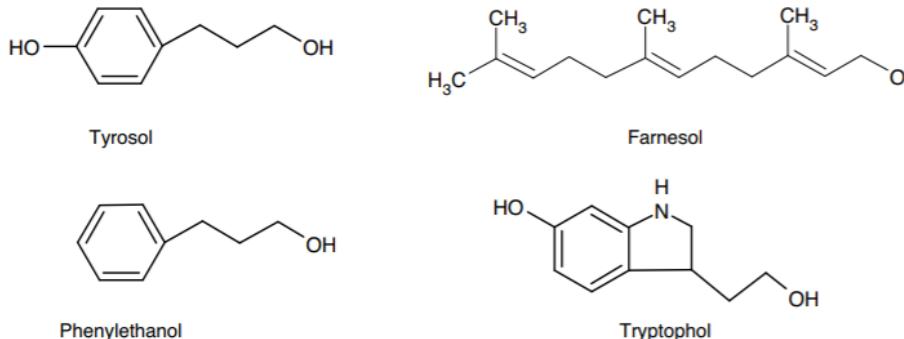
QS was first discovered in the 1970s in the marine luminescent bacterium *Vibrio fischeri*, a facultative symbiont of marine animals such as *Euprymna scolopes* – now more than 70 QS systems have been reported in bacteria

Quorum sensing molecules - examples

Prokaryotes (a)



Eukaryotes (b)



International Journal of Food Microbiology 135 (2009) 299–302



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RESEARCH ARTICLE

Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*

Klaus Gori¹, Peter B. Knudsen², Kristian F. Nielsen², Nils Arneborg¹ & Lene Jespersen¹

¹Department of Food Science, Food Microbiology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark; and ²Department of Systems Biology, Center for Microbial Biotechnology, Technical University of Denmark, Lyngby, Denmark



International Journal of Food Microbiology 149 (2011) 269–273



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Short communication

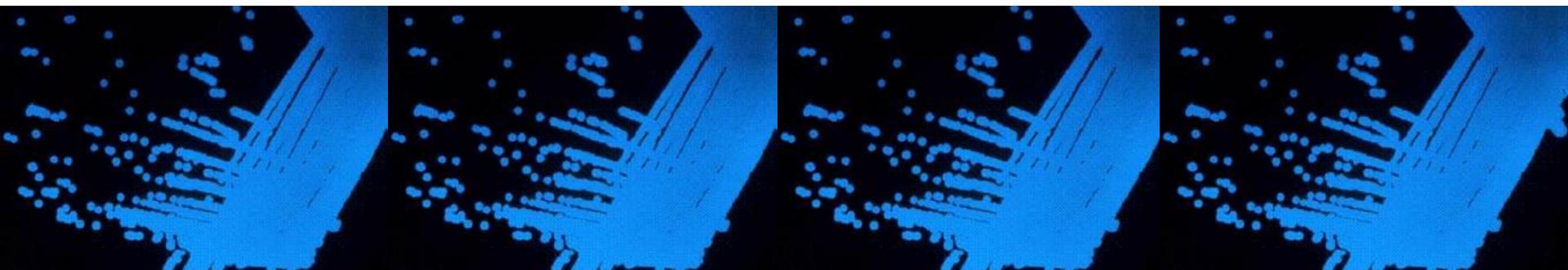
The quorum sensing *luxS* gene is induced in *Lactobacillus acidophilus* NCFM in response to *Listeria monocytogenes*

Salomeh Mosleh-Jenabian ^{*}, Finn Kvist Vogensen, Lene Jespersen

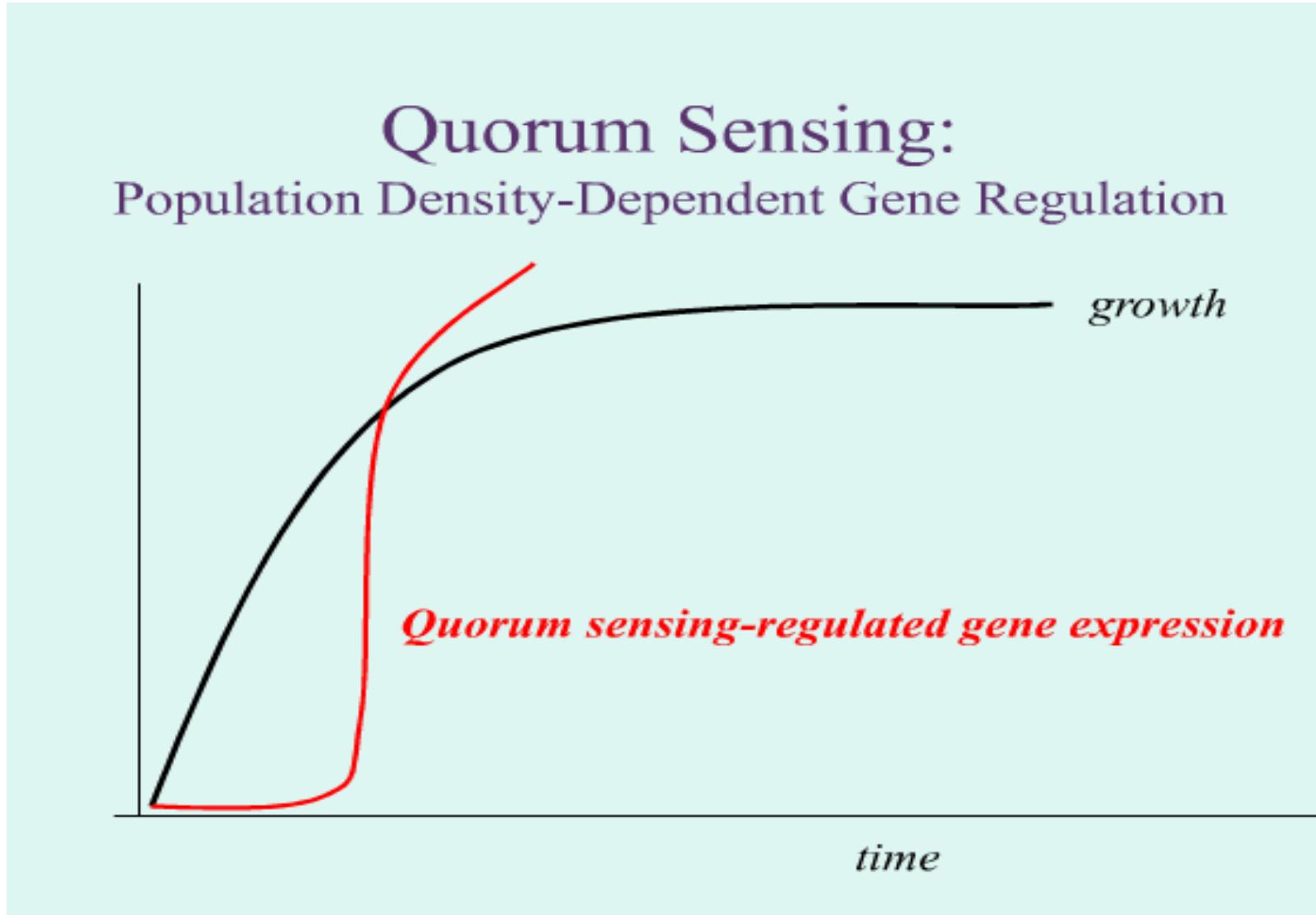
University of Copenhagen, Faculty of Life Sciences, Department of Food Science, Food Microbiology, Rønnevangsgade 30, DK-1958 Frederiksberg C, Denmark

Quorum sensing controlled behaviours

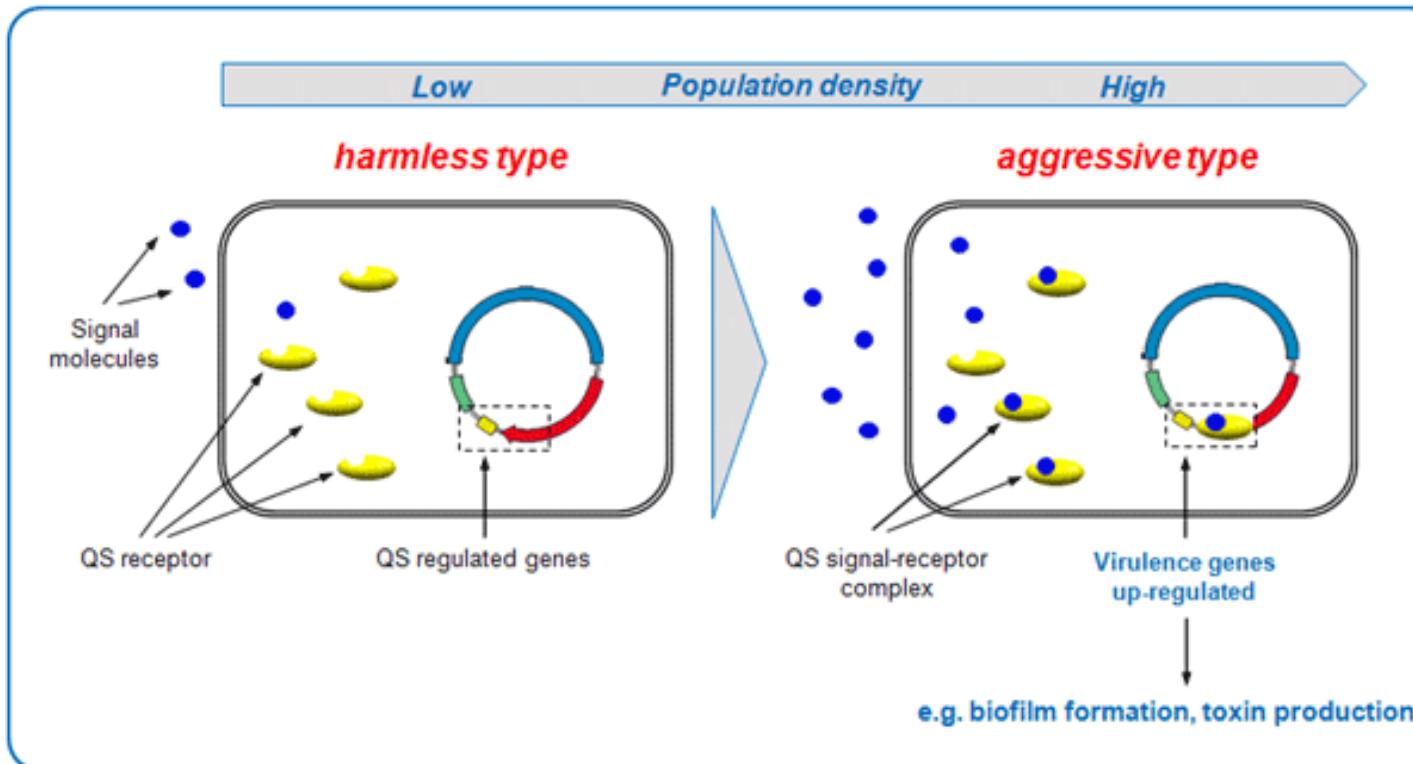
- Virulence
- Pathogenicity
- Toxin production
- Bioluminescence
- Biofilm formation
- Competence for DNA uptake
- Sporulation
- Antibiotic synthesis
- Protease activity
- Nutrient flux
- Carbohydrate metabolism
- Pigment production
- Motility
- Exopolysaccharide (EPS) production
- Iron acquisition
- Cell division
- Bacteriocin synthesis
- Stress tolerance



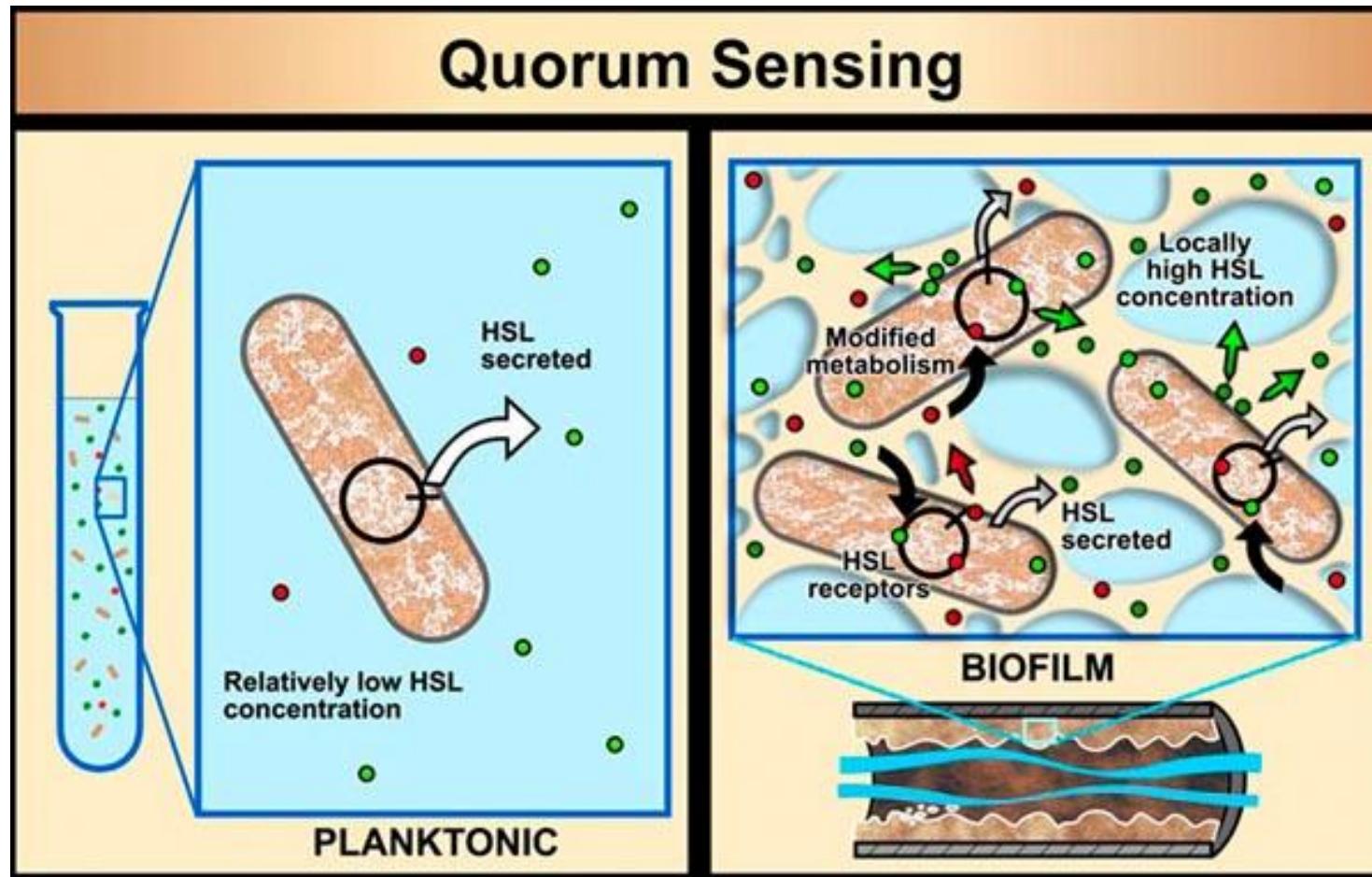
Quorum Sensing and gene expression



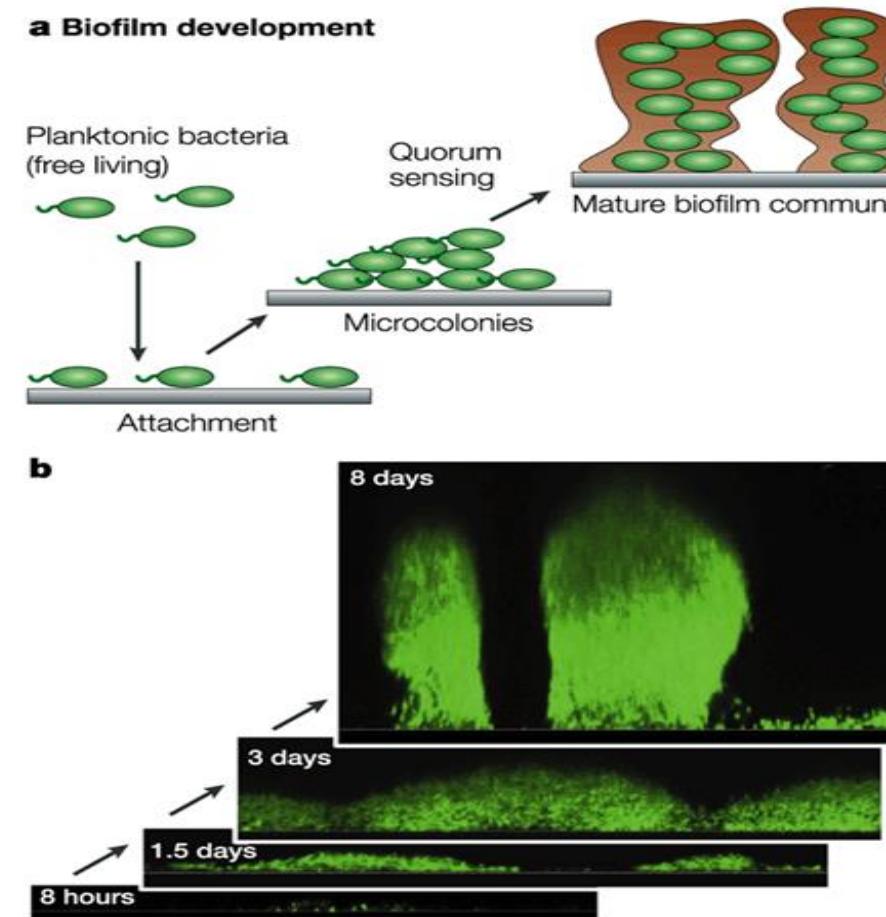
The overall principle of QS (pathogenic bacteria)



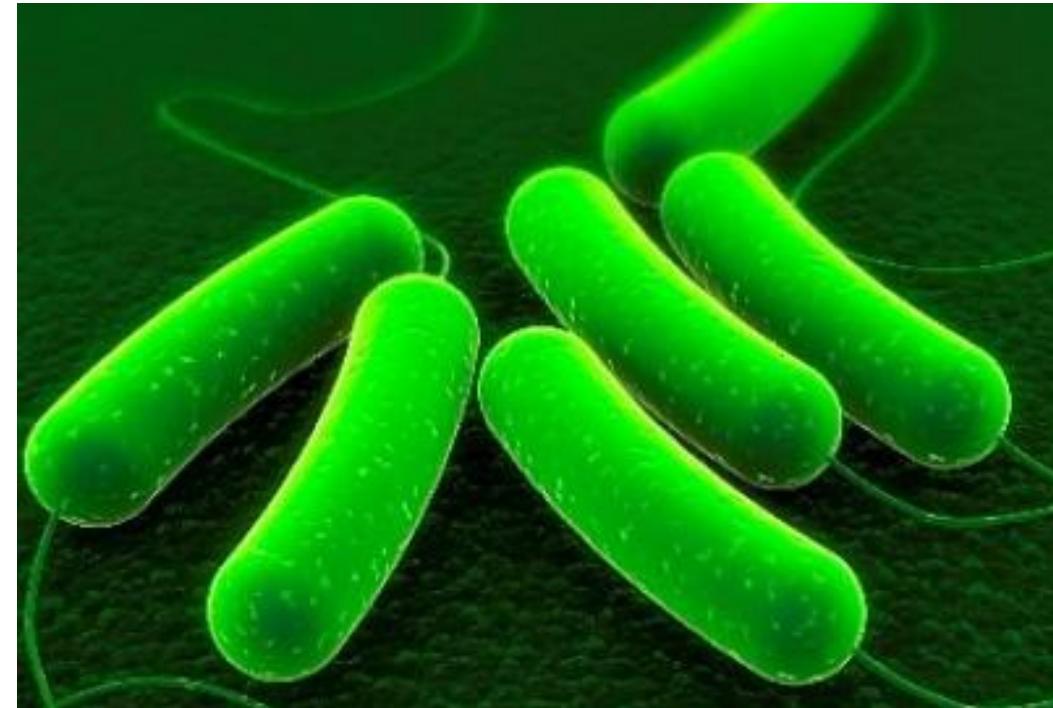
Quorum Sensing in biofilm formation (1/2)



Quorum Sensing in biofilm formation (2/2)



Prokaryotes

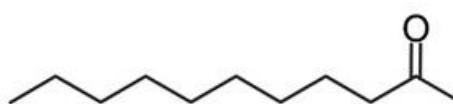
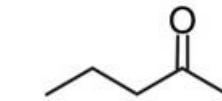
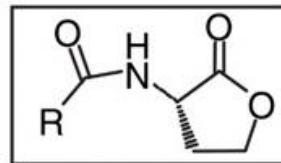


Quorum sensing in bacteria

Intraspecies

Gram-negative bacteria

N-acylhomoserine lactones (AHL)



Intraspecies

Gram-positive bacteria

Peptides (5-17 amino acids)

Val-Ser-Thr-Cys-Asp-Phe-Ile-Met
S. aureus AIP1

Gly-Val-Asn-Ala-Cys-Ser-Ser-Leu-Phe
S. aureus AIP2

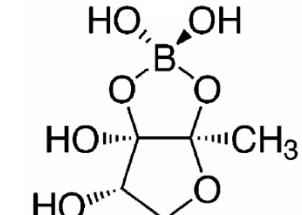
Ile-Asn-Cys-Asp-Phe-Leu-Leu
S. aureus AIP3

Tyr-Asn-Cys-Asp-Phe-Leu-Leu
S. aureus AIP4

Interspecies

Gram-negative and Gram-positive bacteria

Autoinducer-2 (AI-2)

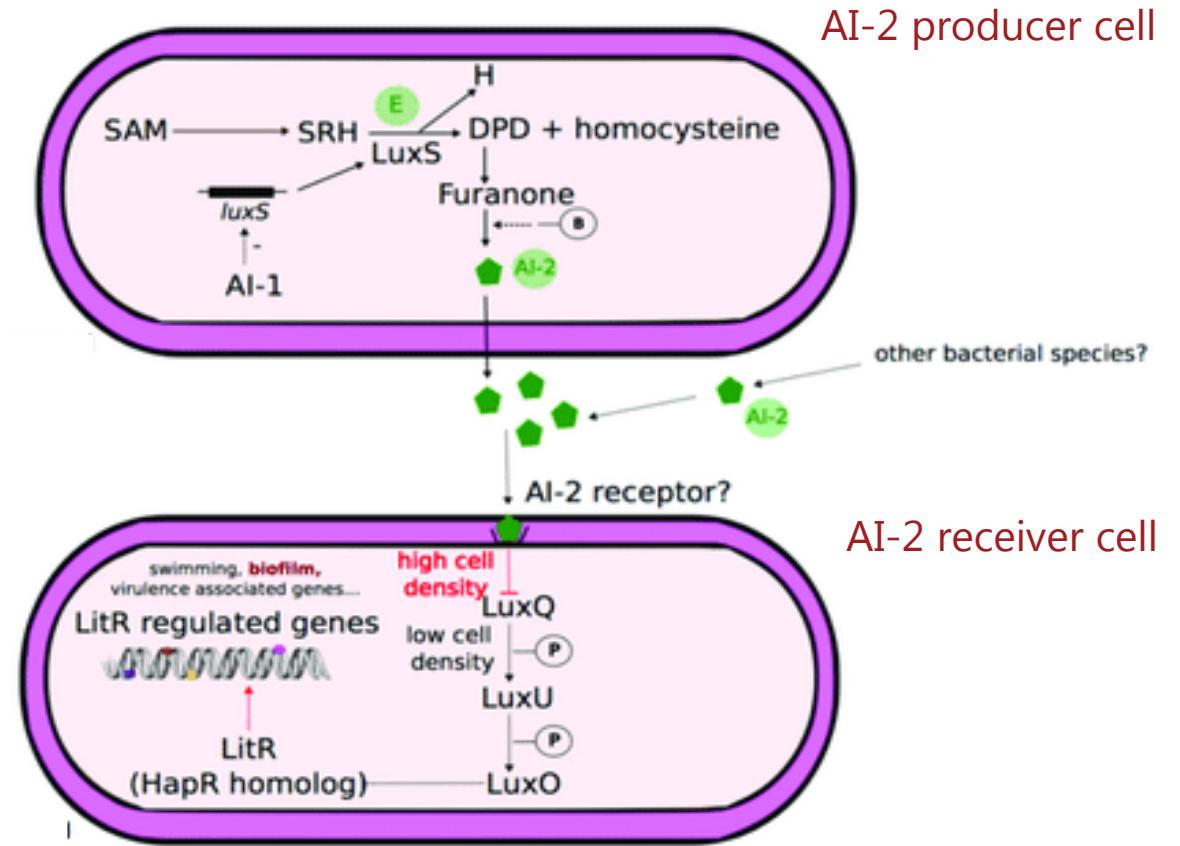
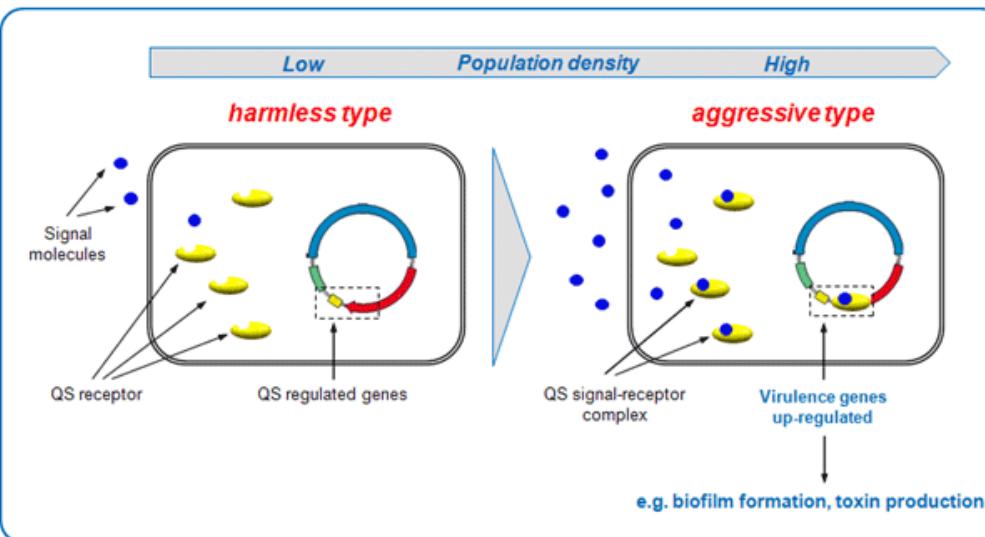
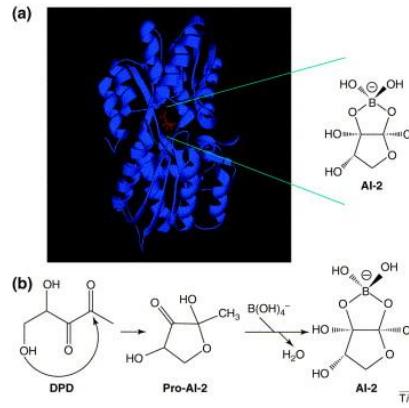


V. harveyi

(2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate

Various forms exist between different bacterial species – are generally rather unstable.

luxS-mediated QS in prokaryotes (interspecies – AI-2)

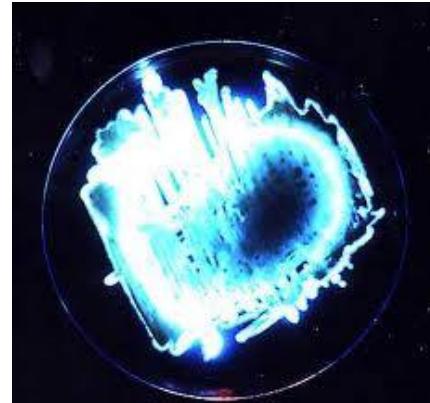


Traits controlled by *luxS*-mediated QS (examples):

- Virulence gene expression (*E. coli* STEC); motility (*Campylobacter jejuni*);
- Biofilm formation (*Listeria monocytogenes*);
- Tolerance to acid and oxidative stress (*Streptococcus mutans*)

AI-2 can be measured by an autoinducer bioassay

- AI-2 activity is usually determined by a bio-assay using *Vibrio harveyi* as sensor organism
- *Vibrio harveyi* will at a threshold level of AI-2 transcribe a luciferase-encoding gene that will induce bioluminescence
- The bio-assay includes:
 - a reporter strain (BB170 – do not produce AI-2)
 - a positive strain (BB152) for standardisation of the assay
 - a negative control (sterile culture medium)
- Supernatants from the bacteria of interest is then added to the reporter strain and the bioluminescence is measured

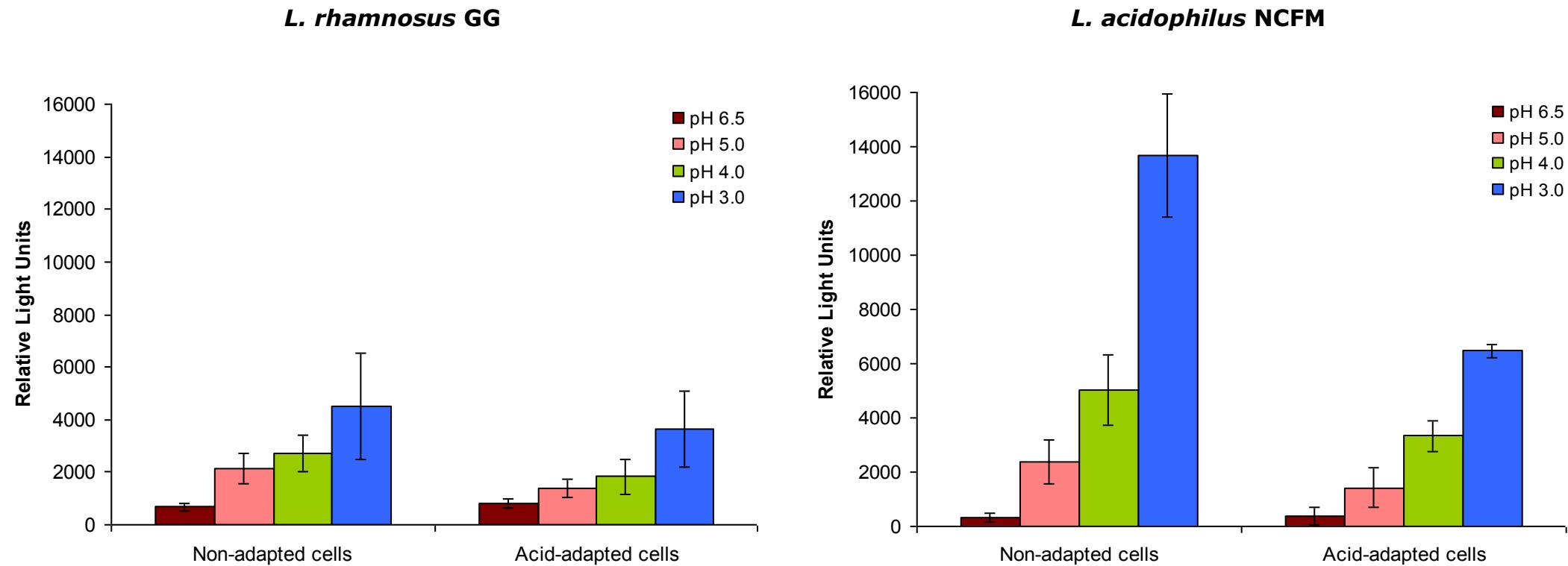


Autoinducer-2 (AI-2) activity of smear bacteria

Strain	Relative AI-2 activity (%) [*]
<i>Microbacterium barkeri</i> 20145	92 ± 5
<i>Arthobacter nicotianae</i> 20123	81 ± 10
<i>Corynebacterium ammoniagenes</i> 20306	78 ± 9
<i>Corynebacterium casei</i> 44701	76 ± 6
<i>Microbacterium gubbeenense</i> 15944	63 ± 8
<i>Staphylococcus equorum</i> subsp. <i>linens</i> 15097	60 ± 14
<i>Corynebacterium ammoniagenes</i> 20305	47 ± 11
<i>Brevibacterium casei</i> 20657	0
<i>Brevibacterium linens</i> BL2	0
<i>Brevibacterium linens</i> B1	0
<i>Brevibacterium linens</i> B2	0
<i>Brevibacterium linens</i> B3	0
<i>Brevibacterium linens</i> M18	0

*Sterile growth medium was set to 0% AI-2 activity, whereas *Vibrio harveyi* BB152 supernatant was set to 100% AI-2 activity

AI-2 induction in *Lacticaseibacillus rhamnosus* and *Lactobacillus acidophilus* at decreased pH values



AI-2 production is higher at low pH and lower in acid-adapted strains

International Journal of Food Microbiology 135 (2009) 295–302



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AI-2 signalling is induced by acidic shock in probiotic strains of *Lactobacillus* spp.

Saloomeh Moslehi-Jenabian *, Klaus Gori, Lene Jespersen

Department of Food Science, Food Microbiology, Faculty of Life Sciences, University of Copenhagen, Rølighedsvej 30, DK-1958 Frederiksberg C, Denmark

luxS expression is upregulated in *Lactobacillus acidophilus* upon inoculation with *Listeria monocytogenes*

International Journal of Food Microbiology 149 (2011) 269–273

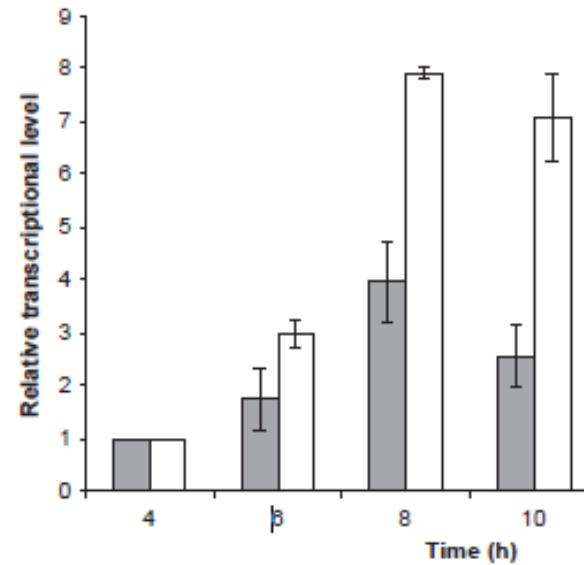


Fig. 3. Transcriptional levels of *luxS* in *L. acidophilus* NCFM grown in mono- (■) and co-culture (□) with *L. monocytogenes* in MRS/BHI medium incubated at 37 °C.

Important note: *L. monocytogenes* has been reported to posses *luxS* genes



Short communication

The quorum sensing *luxS* gene is induced in *Lactobacillus acidophilus* NCFM in response to *Listeria monocytogenes*

Saloomeh Mosleh-Jenabian *, Finn Kvist Vogensen, Lene Jespersen

University of Copenhagen, Faculty of Life Sciences, Department of Food Science, Food Microbiology, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

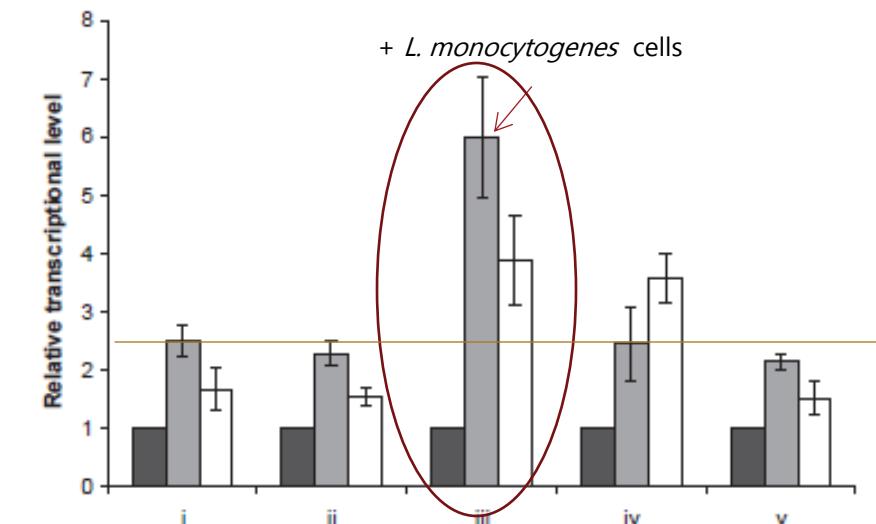
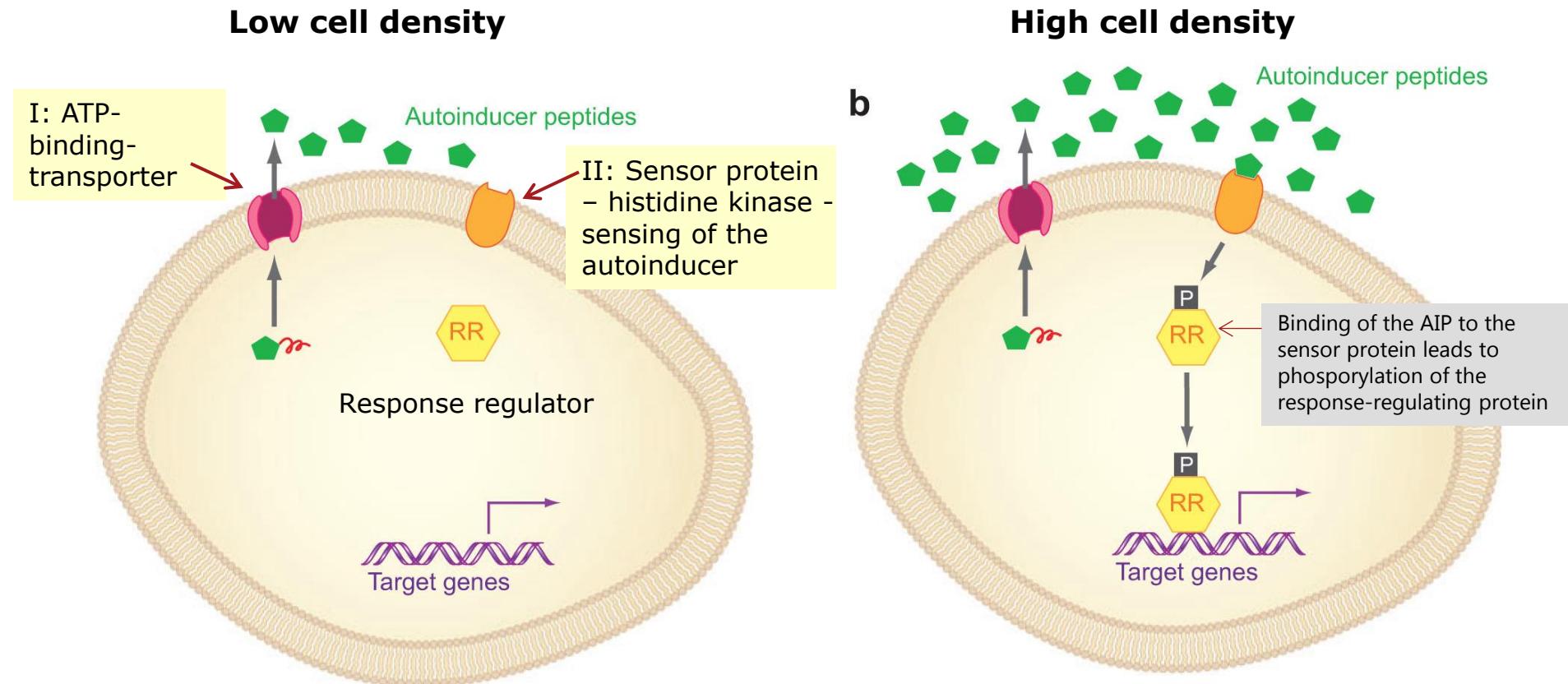


Fig. 4. Transcriptional levels of *luxS* in *L. acidophilus* NCFM mid-exponential growing cells after 0 h (■), 2 h (□) and 4 h (□) incubation alone (i), or with the same number of its own cells (ii), *L. monocytogenes* cells (iii), *L. monocytogenes* sterile pH adjusted cell-free culture medium (iv) or *L. monocytogenes* heat-killed cells (v).

Production of bacteriocins (some are autoinducer peptides = AIPs)

- Bacteriocins are antimicrobial peptides primarily produced by Gram-positive bacteria, in particular lactic acid bacteria. Bacteriocins have a potential application as biopreservatives in food systems
- In many microorganisms, bacteriocin production requires secretion and extracellular accumulation of peptides that act as chemical messengers (QS) and trigger bacteriocin production
- The autoinducer peptides (AIPs) are often referred to as autoinducers being able of QS-based regulation of bacteriocin production
- Notably, the peptides acting as AIPs can be with or without antimicrobial activity

The two-component type QS mechanism AIPs (Gram positive bacteria - intraspecies)

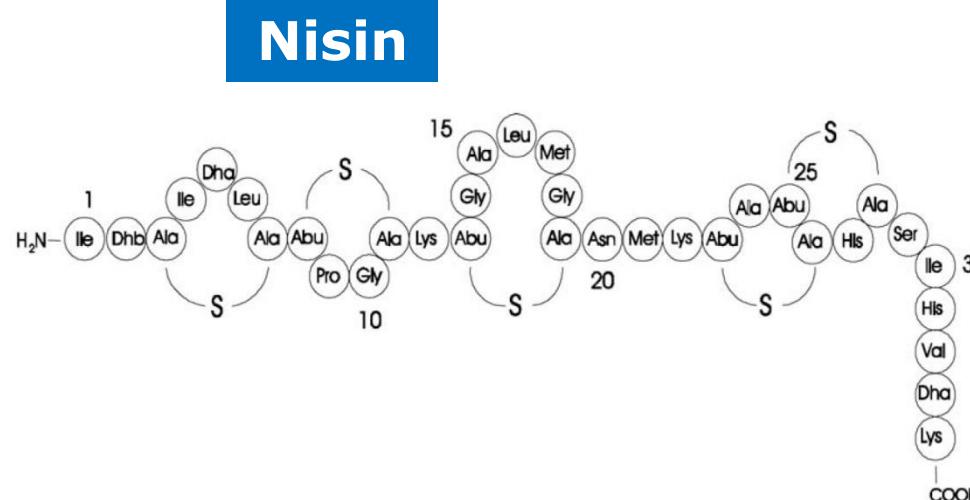


Traits controlled by the 2-component systems (examples):

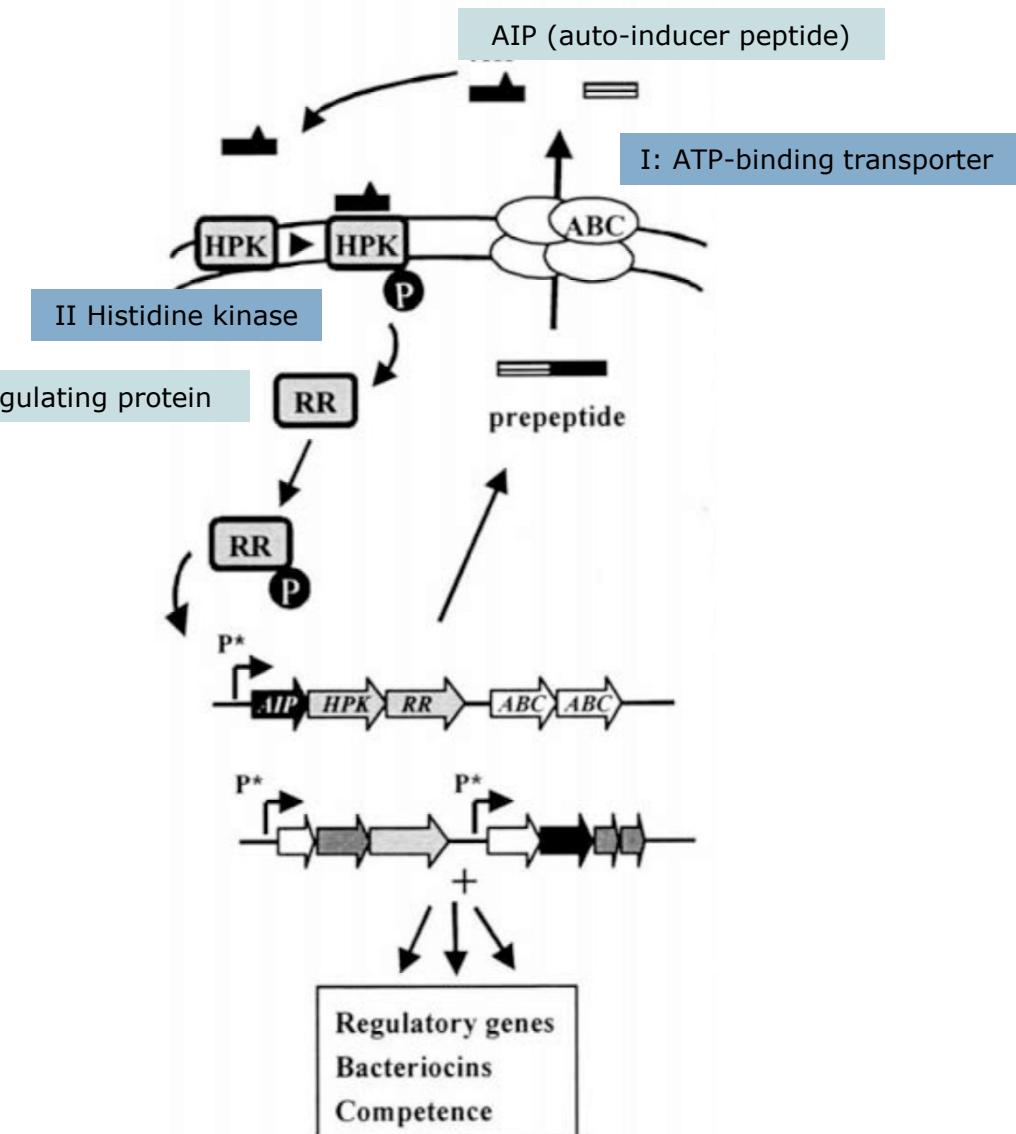
- Bacteriocin production (*Lactiplantibacillus plantarum*, *Lactobacillus acidophilus* and *Ligilactobacillus salivarius*)
- Genetic competence - the ability to take up high-molecular-weight exogenous DNA i.e. transformation (*Streptococcus pneumoniae* and *Bacillus subtilis*)
- Sporulation (*B. subtilis*)
- Virulence gene expression (*Staphylococcus aureus*)

Peptides as Quorum Sensing molecules in Gram-positive bacteria

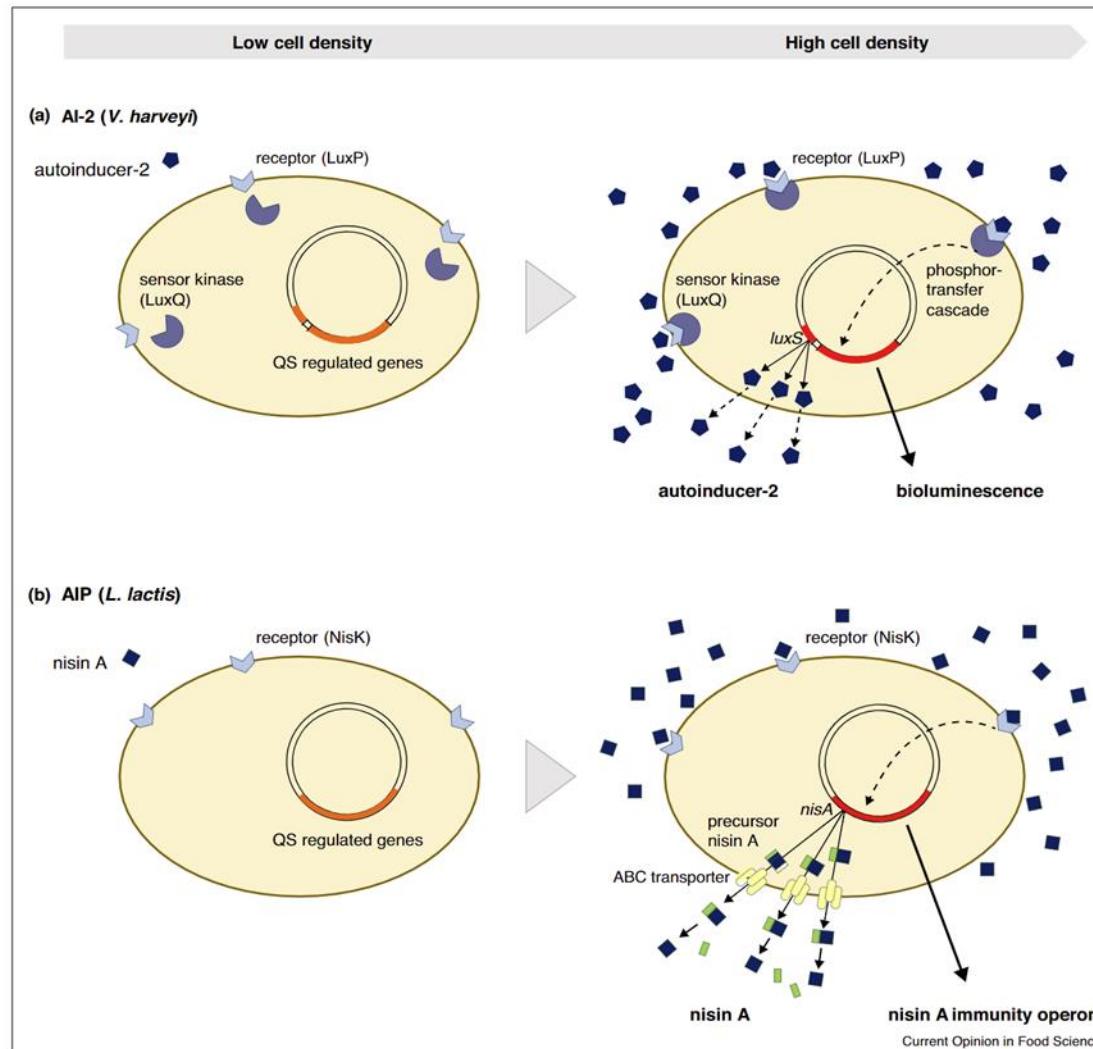
The antimicrobial peptides also exhibit a peptide pheromone function that plays an essential role in QS control of their biosynthesis



Subtilin production in *Bacillus subtilis* is autoregulated in the same manner as nisin in *Lactococcus lactis*



Different QS regulation mechanisms exist – but the basic principles are the same



Illustrations of two quorum sensing (QS) systems. (a) Autoinducer-2 (AI-2) dependent QS in *Vibrio harveyi*; at high cell densities AI-2 binds to a receptor protein (LuxP) which interacts with a sensor kinase (LuxQ). This complex initiates a phosphor-transfer cascade inducing transcription of the bioluminescence genes and *luxS* (involved in the synthesis of AI-2). (b) Autoinducer peptide (AIP) dependent QS for nisin A in *Lactococcus lactis*; at high cell densities nisin A binds to sensor kinase proteins (NisK) that interact with response proteins, which induce transcription of the QS regulated target genes. *nisA* encodes the precursor peptides, which are secreted via ATP-binding cassette (ABC) transporters. Outside the cells the leader sequence is removed resulting in mature nisin A with antimicrobial activity. The nisin A immunity operon includes genes protecting the producing *L. lactis* cells from the antimicrobial activity of nisin A.

- a) **AI-2 *Vibrio harveyi***
- b) **AIP *Lactococcus lactis***

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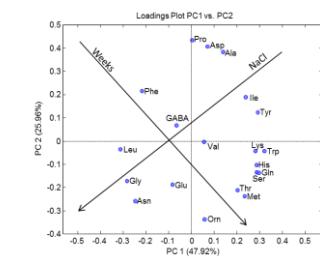
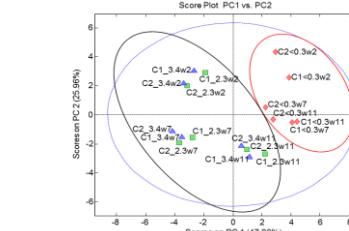
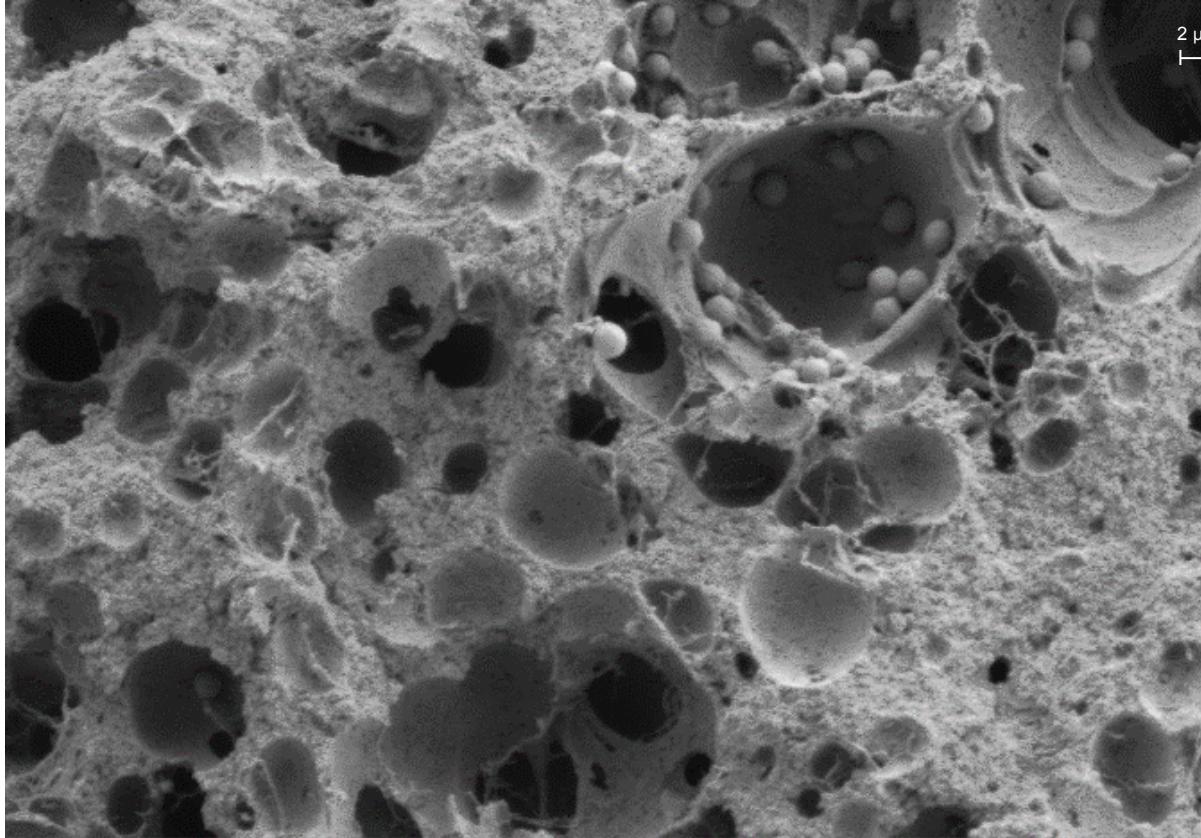
Current Opinion in
Food Science

Impact of quorum sensing on the quality of fermented foods
Pernille Johansen and Lene Jespersen

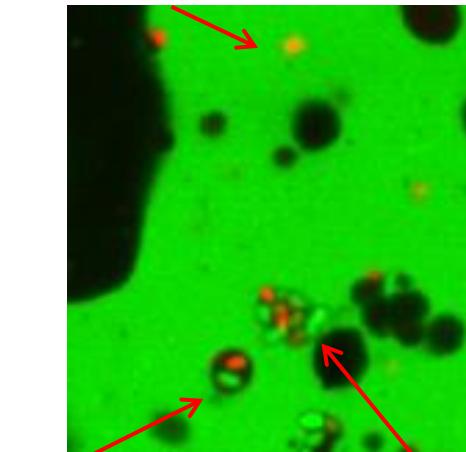


We do not know how many functions that are under QS control!

Does colony size influence autolysis?



Single dead cell



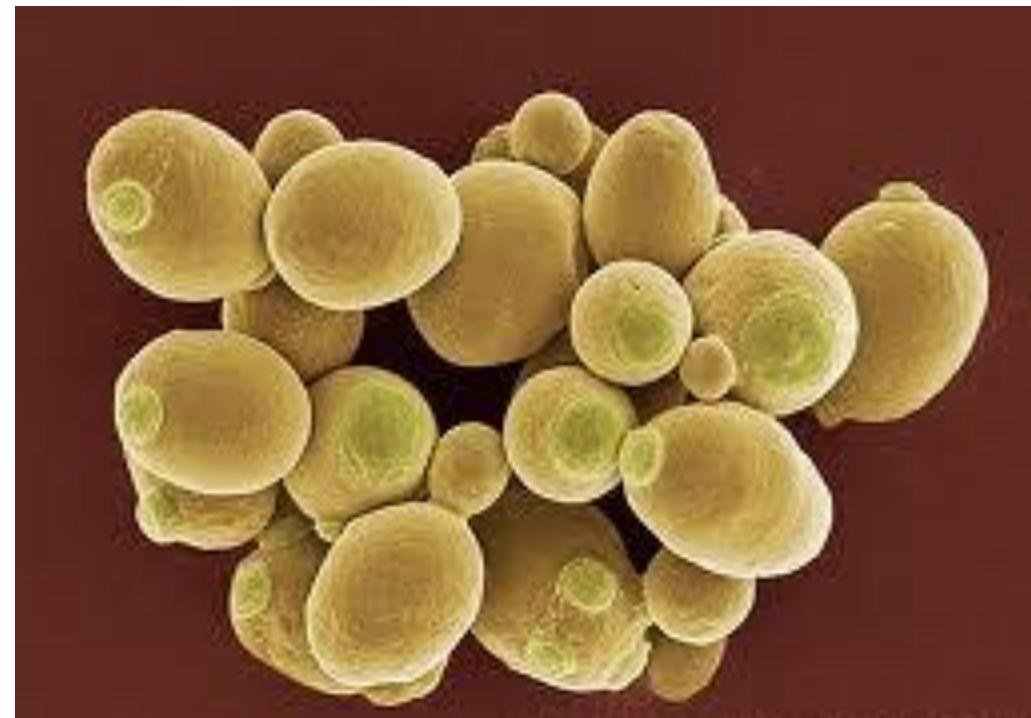
Impact of NaCl reduction in Danish semi-hard Samsøe cheeses on proliferation and autolysis of DL-starter cultures
Lise Sandsgaard^{a,*}, Mia Rysel^b, Carina Svendsen^a, Erik Høier^b, Ulf Andersen^c, Marianne Hammershøj^d, Jean R. Müller^{d,1}, Nils Amebørg^d, Lene Jespersen^d
^a Department of Food Science, Faculty of Science, University of Copenhagen, 1958 Frederiksberg C, Denmark
^b Chr. Hansen AS, 2670 Brænderup, Denmark
^c Chr. Hansen AS, 2670 Brænderup, Denmark
^d Department of Food Science, Aarhus University, Birkens Allé 20, Postboks 10, 8000 Århus, Denmark

CHR HANSEN

Arla

- The cheese NaCl content had a significant culture-dependent influence on proliferation, viability and autolysis of the DL starter cultures
- During ripening, loss of viability and autolysis were most pronounced for bacteria in groups of ≥ 4 bacteria (is autolysis under QS control?)**

Eukaryotes

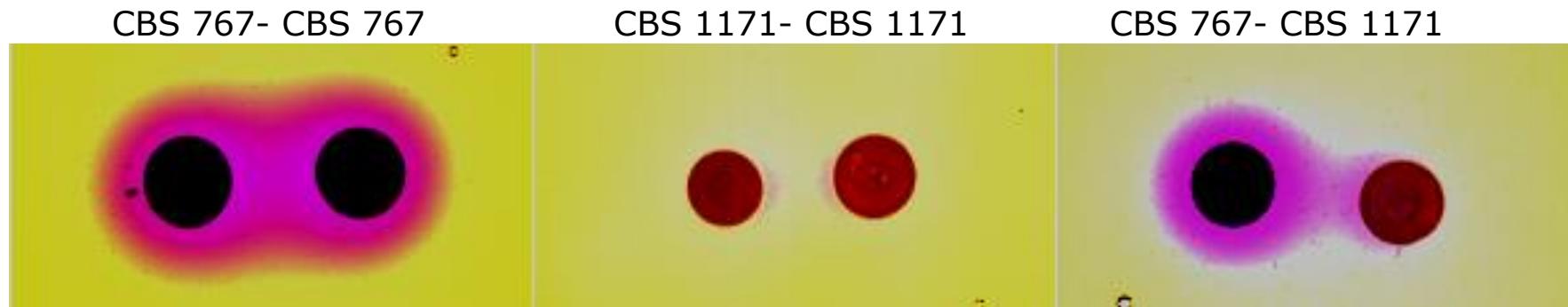


Small talk – cell to cell signalling

- As for prokaryotes, intra – and interspecies communication among eukaryotes might take place in complex food environments
- In yeasts, signalling molecules are used to induce behaviours such as growth, virulence, biofilm formation, sporulation, NaCl tolerance etc.

Colony directed sensing leads to cell directed growth and selective cell death

S. cerevisiae meets *D. hansenii*!

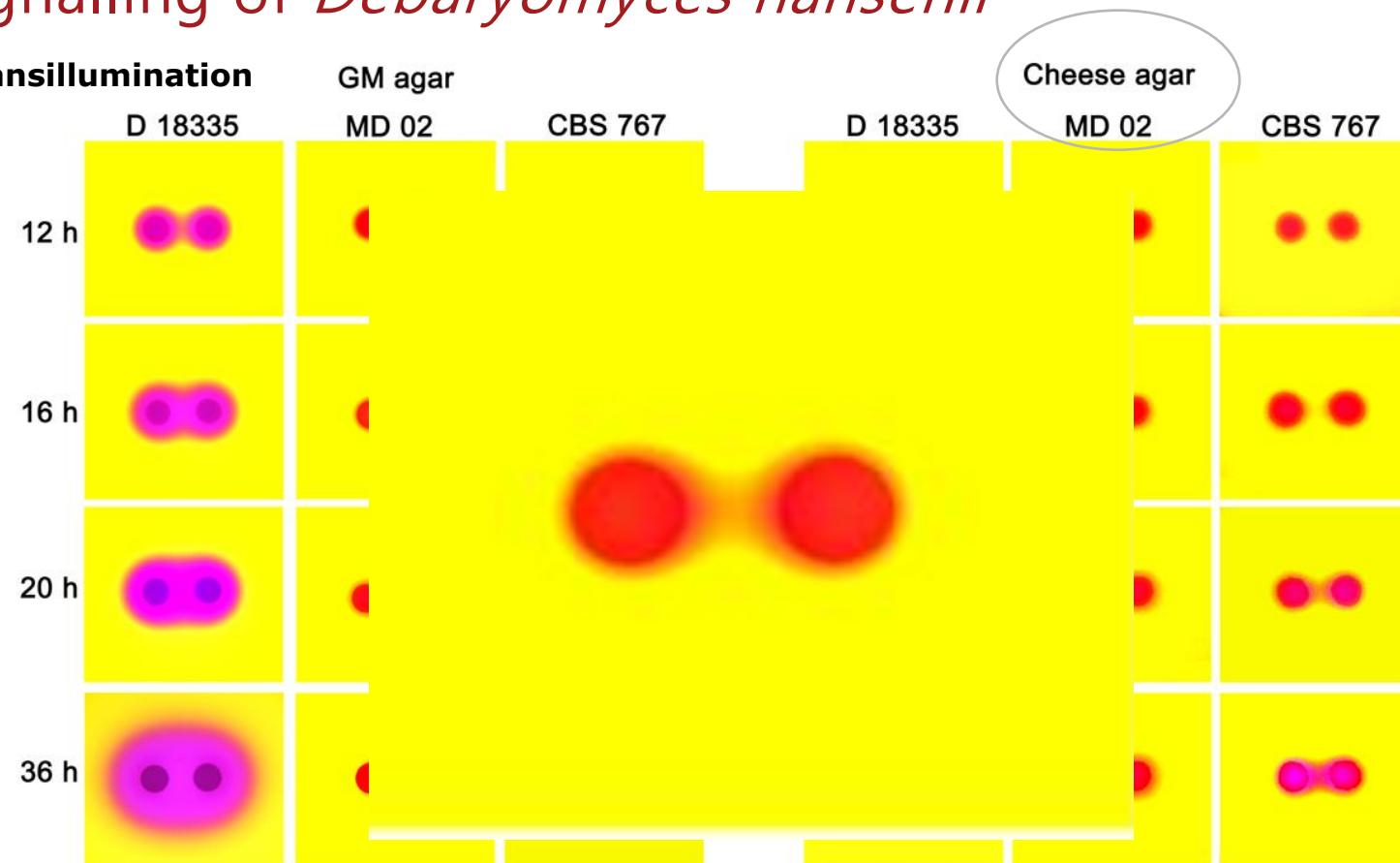


CBS 767: type strain of *D. hansenii*; CBS 1171: type strain of *S. cerevisiae*

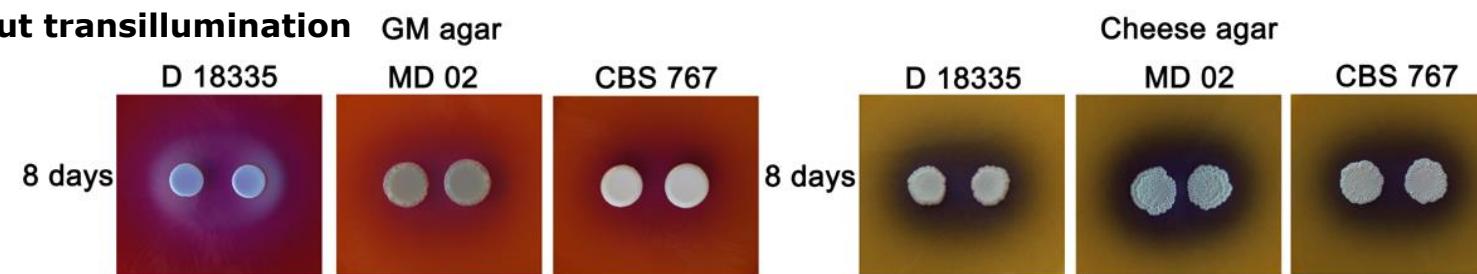
GM agar contains the pH indicator bromcresol purple. pH < 5.2: yellow, pH > 6: violet

Ammonia signalling of *Debaryomyces hansenii*

With transillumination

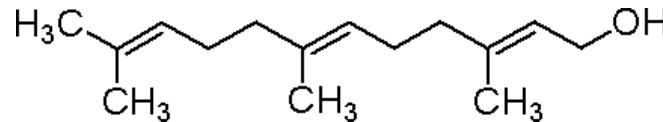


Without transillumination



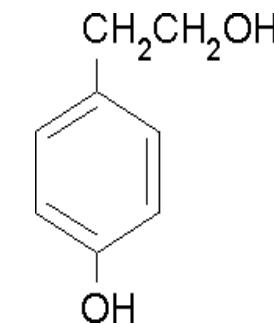
Alcohol-based quorum sensing in yeasts

Candida albicans



Farnesol

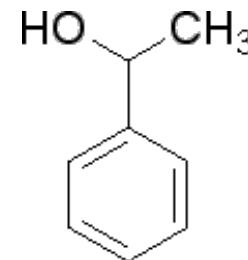
Filamentation and biofilm formation ↓



Tyrosol

Filamentation ↑ Lag phase ↓

Saccharomyces cerevisiae

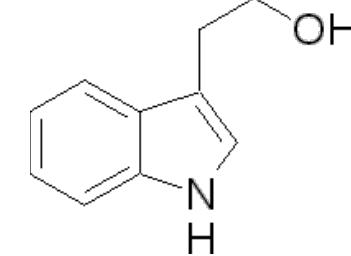


Phenylethanol

Pseudohyphal growth ↑

Invasive growth ↑

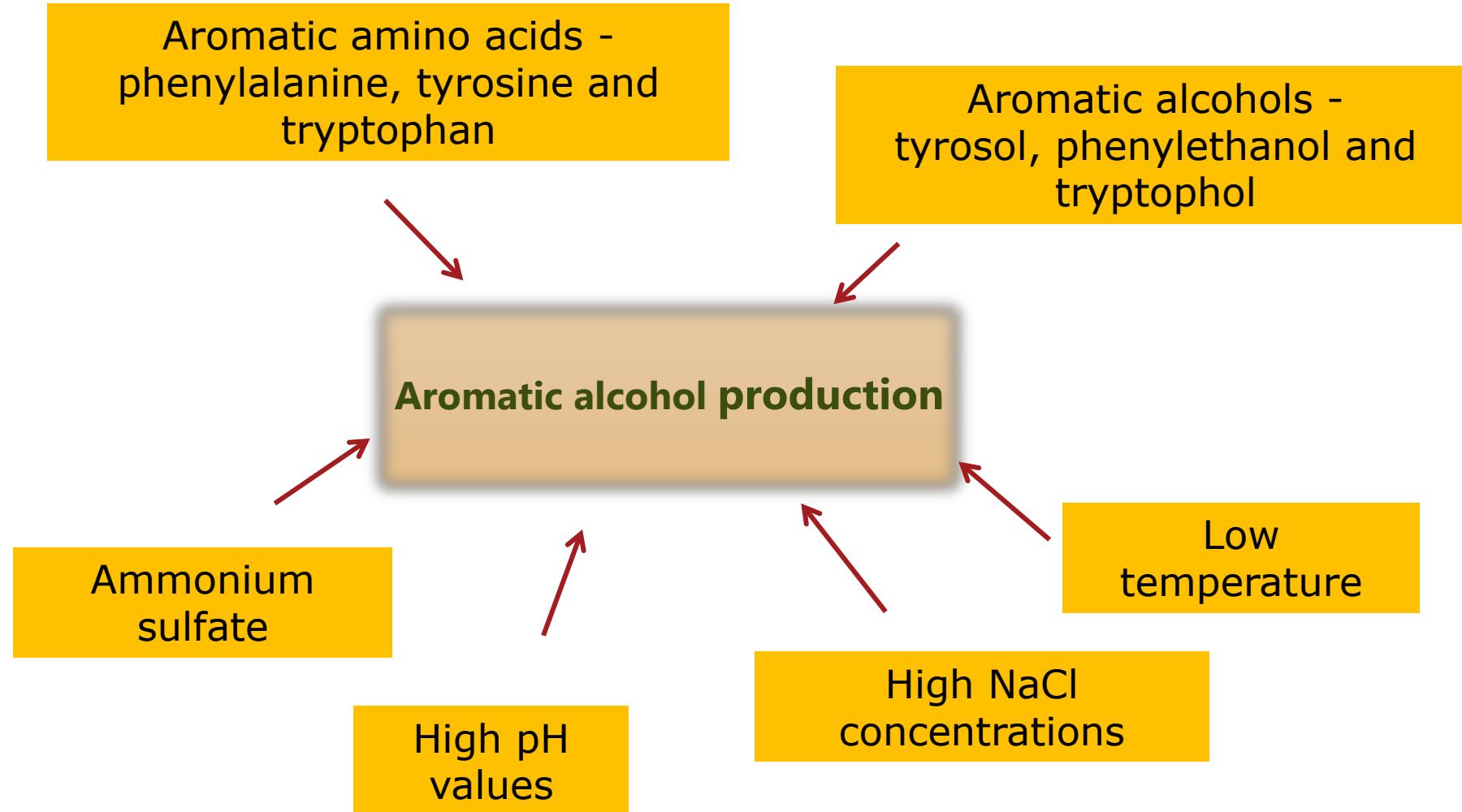
Even greater in presence of tryptophol



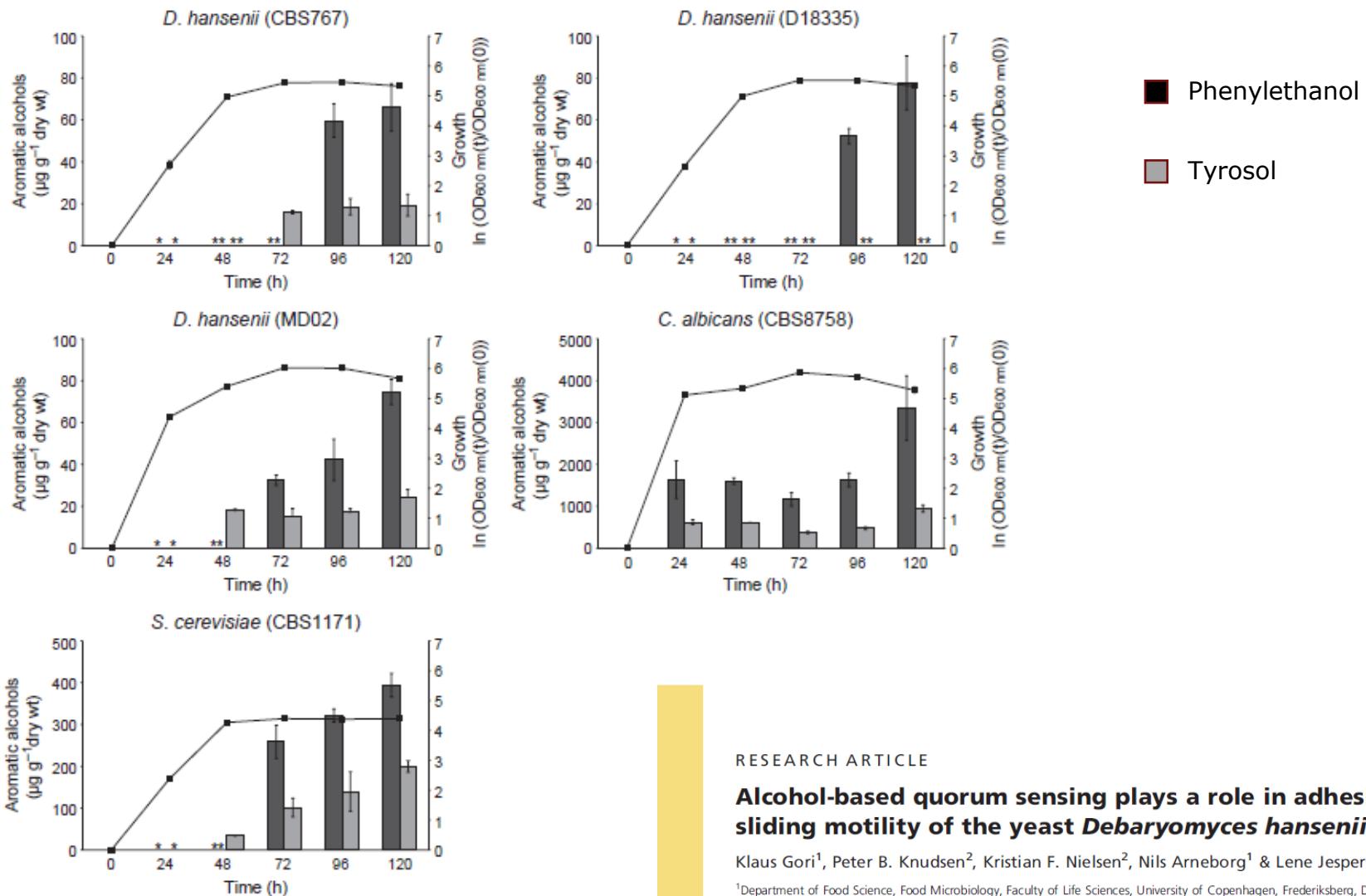
Tryptophol

Pseudohyphal growth ↑

Environmental conditions influencing aromatic alcohol production



Quorum sensing molecules are produced by several food related yeast species – e.g. *D. hansenii*



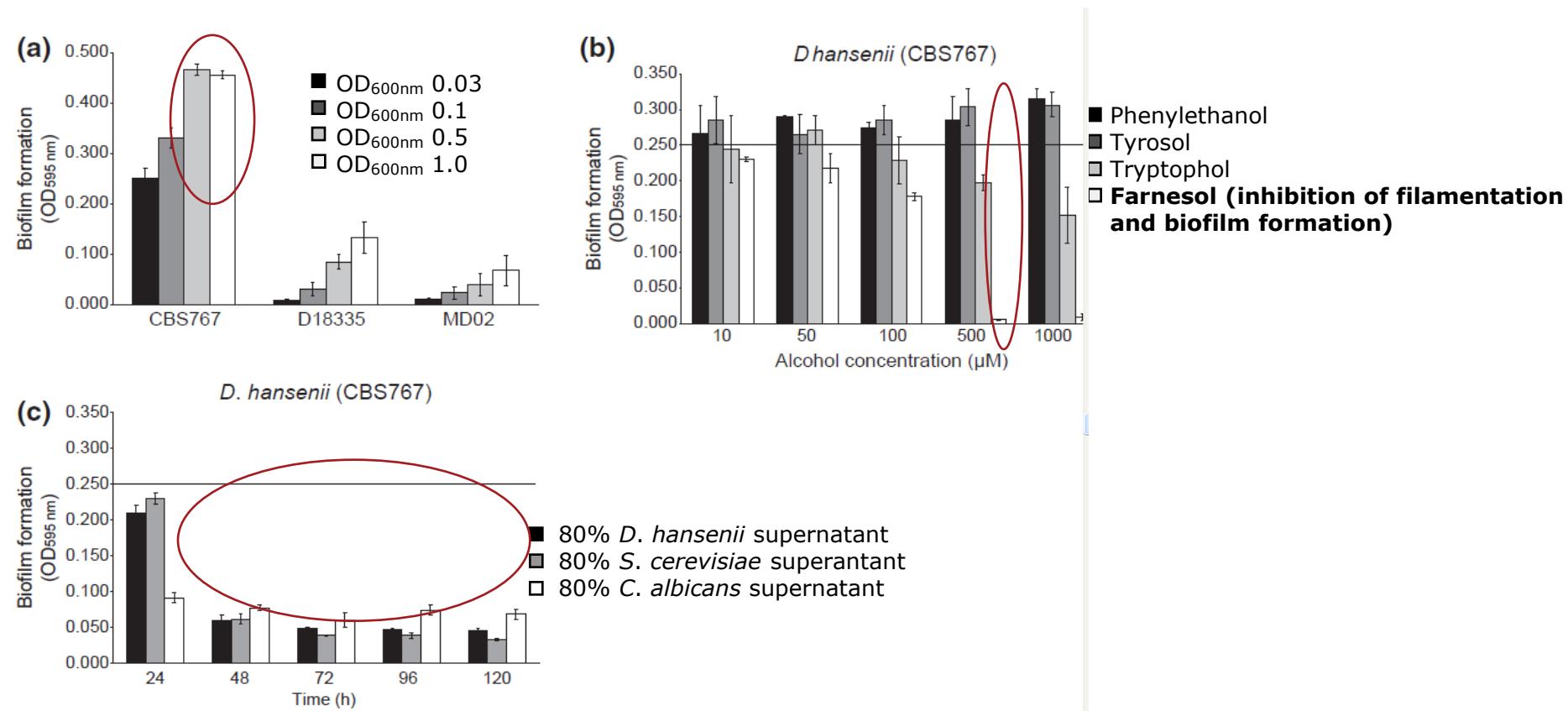
RESEARCH ARTICLE

Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*

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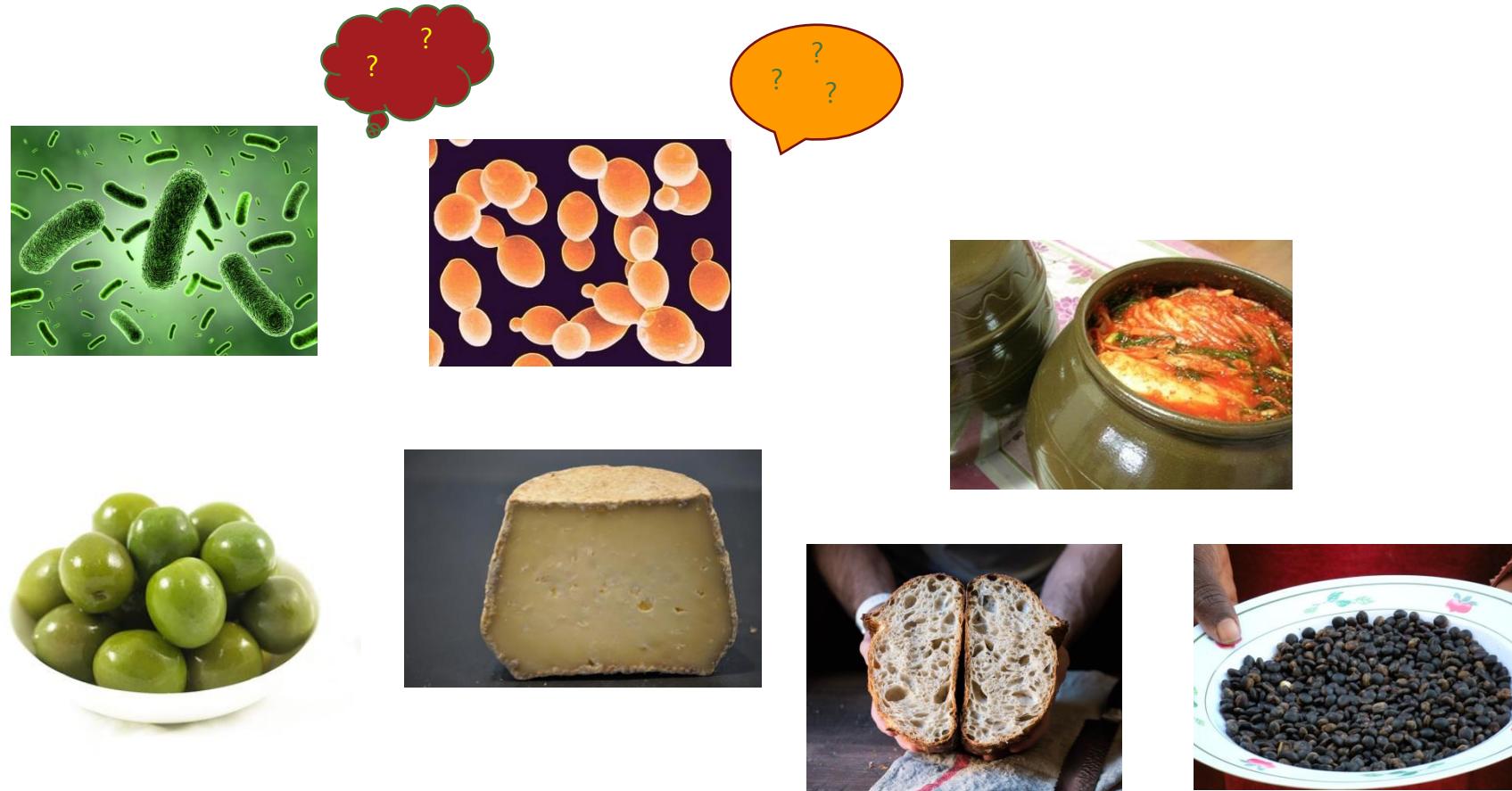
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Quorum sensing molecules influence adhesion and biofilm formation of *Debaryomyces hansenii*



Adhesion and biofilm formation of *D. hansenii* is strain dependent and influenced by (a) growth phase, (b) quorum sensing molecules and (c) supernatants from other yeast species

Quorum sensing in fermented food and beverages



ELSEVIER

Impact of quorum sensing on the quality of fermented foods

Pernille Johansen and Lene Jespersen

Current Opinion in Food Science 2017, 13:16–25

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Current Opinion in
Food
Science



Quorum sensing within microbial communities of fermented foods

QS within microbial communities in fermented foods				
Food matrix	Microorganism(s)	QS system	QS effect	Reference
Vegetable based				
Kimchi	LAB			
Spanish-style green olive	LAB			
Spanish-style green olive	LAB			
Fermented vegetables	LAB			
Chinese fermented cabbage	LAB			
Carrot, tomato, pineapple	LAB			
Sourdough				
Sourdough	LAB			
Sourdough	LAB			
Alkaline fermented foods				
Seed condiments	AEB			
Dairy-based				
Smear ripened cheese	Gram coryn staph			
Smear ripened cheese	Yeast			
Smear ripened cheese	Yeast			
Dairy-based media	LAB, O			
Cheese matrix	LAB			
Meat				
Chinese fermented meat	LAB			
Wine				
Synthetic wine must	Yeasts			
Synthetic wine must	Yeast			
Probiotics				
	Probiotic LAB	AI-2	AI-2 activity found in <i>L. rhamnosus</i>	[30]
	Probiotic LAB	AI-2	AI-2 activity found in <i>L. rhamnosus</i> , <i>L. salivarius</i> , <i>L. acidophilus</i> and <i>L. johnsonii</i> .?AI-2 activity and transcription of <i>luxS</i> were induced upon acid shock	[10]
	Probiotic LAB	AI-2	<i>luxS</i> transcription in <i>L. acidophilus</i> was induced upon co-cultivation with <i>L. monocytogenes</i> and growth of <i>L. monocytogenes</i> was reduced	[57]

Abbreviations: LAB, lactic acid bacteria; AEB, aerobic endospore-forming bacteria; AIP, autoinducer peptide; AI-2, autoinducer 2; PEOH, phenylethanol; TYR, tyrosol; TOL, tryptophol; *luxS*, AI-2 synthesis gene, FAR, farnesol.

- QS reported fermented foods:

Food matrices:

- Vegetable based foods (*Lactiplantibacillus plantarum*, *P. pentosaceus* etc.)
- Sourdough (*L. plantarum*)
- Alkaline fermented foods (*Bacillus subtilis*)
- Dairy-based foods (*D. hansenii*, *G. candidum*, *S. cerevisiae*, *Y. lipolytica*)
- Meat (*Latilactobacillus sakei*)
- Wine (*H. uvarum*, *S. cerevisiae*, *T. pretoriensis*, *Z. bailii*)
- "Probiotics" (*Lacticaseibacillus rhamnosus* etc.)



Why is Quorum Sensing (QS) of interest for food microbiologists ?

- QS might be of importance for regulation of complex microbial communities occurring in food and beverages
- QS might regulate important technological properties or be of importance for the survival of the cultures under stressful conditions
- QS is involved in the production of some antimicrobials/some bacteriocins
- QS is for many microorganisms involved in the regulation of biofilm formation
- QS might provide new antibiotic compounds

Remaining scientific questions

- Why is Quorum Sensing (QS) predominant in some species ?
- Which genes are under QS control – and why ?
- Is QS relevant in solid stage fermentations ?
- Can several QS molecules be used in the food industry ?
- Is QS relevant in controlling our gut microbiota ?
- Does probiotic/host cross-signaling exists ?
- Can we prevent illness caused by foodborne pathogens by use of QS blockers ?
- Can “probiotic” microorganisms act as QS blockers by taking up autoinducers in the gut?
- Are there any yet unknown sensing systems ?
- Do cross-kingdom QS interactions exist ?
-???

TECHNOLOGY FEATURE STOP THE MICROBIAL CHATTER

Bacteria can coat everything from thermal springs to teeth. Researchers are looking for antibiotics that can subvert the signalling that the microbes use to carve their niche.



Sheets of communicating bacteria—or biofilms—are a common sight in the sun-dappled crevices from hot springs in Yellowstone National Park.
BY VIVIEN MAYER

Bacteria are continually producing ways to try to find ways to subvert the microbes by interfering with the signals they use to communicate.

To understand the microbial language, scientists first need to work out what they are saying. Bacteria use chemical signals to encourage others to grow, sense microorganisms as

leaving up with chemists and engineers to try to find ways to subvert the microbes by interfering with the signals they use to communicate.

Such molecular coordination is thought to be crucial for the success of bacteria. Many

of bacteria that spread across surfaces such as hospital catheters or water filtration systems. Some of the bacteria in a biofilm suspend

their metabolism, explains microbiologist J

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Take home messages

- ✓ Microbial sensing regulates gene expression of many microorganisms. Quorum Sensing (QS) is the most well documented form for sensing
- ✓ QS regulates many different traits in both pro- and eukaryotes. The most well-described are virulence, biofilm formation, motility, bacteriocin production, sporulation etc.
- ✓ Recent studies have shown that QS is also relevant for foodborne microorganisms and for probiotics and may also be relevant for the control of spoilage microorganisms
- ✓ For foodborne microorganisms, QS has mostly been studied for stress responses and adhesion properties
- ✓ As an example, cheese ripening cultures produce QS molecules which are up-regulated under dairy-relevant stress conditions such as increased NaCl concentrations, decreased pH etc.
- ✓ In the future, knowledge on QS might be used for optimisation of starter cultures, bio-control and prevention of illnesses caused by food borne pathogens



Faculty of Science

LAB in Foods and Beverages

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Intended learning outcomes

- Overview of LAB in fermented foods and their contributions to the fermentation
- Understanding of the role of LAB in food fermentations
- Reflecting on possible applications of LAB in fermentations



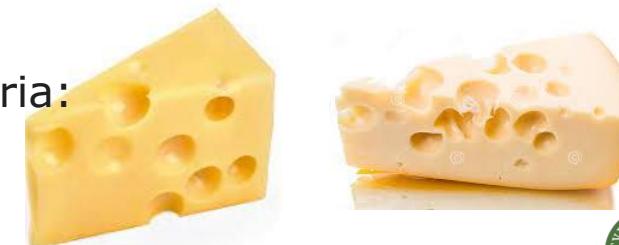
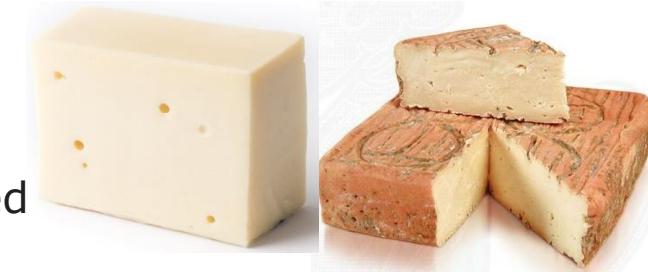
LAB and their roles in fermented foods...



LAB in dairy products

- Best characterised due to long tradition in dairy industry
- Used as starter cultures- added purposefully to perform the fermentation with desired characteristics
 - Defined or undefined
 - Defined composed of few, well-characterised strains
 - Undefined composed of multiple strains with changed proportions over time
 - Mesophilic or thermophilic
 - Mesophilic come from Northern Europe and ferment at 22-25 ° C
 - Thermophilic come from Southern Europe and ferment at 40-42° C

- Ferment alone or in combination with other microorganisms (adjunct cultures)
 - LAB+yeast+ acetic acid bacteria: kefir
 - LAB+red smear (yeasts and corynebacteria/brevibacteria): red smear cheeses like Danbo or Taleggio
 - LAB+mould: Camembert and Brie (white mould) or Danablu and Gorgonzola (blue mould)
 - LAB+ propionic acid bacteria: Emmental, Maasdam



LAB in cheeses (starter culture)

- Thermophilic starter (growth optimum 38-44 ° C)
- e.g. Grana Padano, Emmental, Mozarella

- *Streptococcus thermophilus*
- *Lactobacillus helveticus*
- *Lactobacillus delbrueckii* subsp. *bulgaricus*
- *Lactobacillus delbrueckii* subsp. *lactis*

- Mesophilic starter (growth optimum 25-32 ° C)
- E.g. Camembert, Gouda, Danbo

- *Lactococcus lactis*
- *Lactococcus lactis* biovar. *diacetylactis*
- *Lactococcus cremoris*
- *Leuconostoc mesenteroides* subsp. *cremoris*
- *Leuconostoc lactis*
- *Leuconostoc pseudomesenteroides*

Heterofermentative

The species appear in different combinations depending on the product

- Starter cultures for dairy originated from raw milk (i.e. both animal and farm environment) used for production and differed by geographical location
- Developed and standardised over time to ensure reproducible results



Role of thermophilic starter

- Utilization of ***all lactose** in cheese
 - Lactose/galactose metabolism into lactate
 - S. thermophilus* excretes galactose (antiporter)
 - Lactobacillus helveticus*/*Lactobacillus delbrueckii* subsp. *lactis* will convert galactose to lactate, if present
 - Citrate not converted by thermophilic LAB (no eyes)
- Proteolysis:** together with **milk plasmin** degrade caseins into oligopeptides
 - Cell wall associated proteinases** = Cell Envelope Proteinases = CEP (see Dairy Microbiology)
 - Rennet is normally inactivated at high cooking temperatures (and doesn't play a role anymore)



- Degradation of peptides to amino acids
 - Cell wall-associated peptidases
 - Oligopeptide uptake system
 - Intracellular peptidases
- Conversion of amino acids to aroma compounds

*Most lactose is removed with whey, and the rest is removed by starter over time. The most lactose is left in soft, unripened cheeses like mozzarella (thermophilic) or cottage cheese (mesophilic).

and mesophilic starter in cheeses

- Utilization of ***all lactose** in cheese
 - Lactose metabolism into lactate, acetate, and CO₂
 - Eye formation when wanted
 - Conversion of citrate into diacetyl, acetate, and CO₂
 - Fast conversion (many small holes)
 - Slower conversion (fewer but larger holes)
- Proteolysis:** together with **rennet** and **milk plasmin** degrade caseins into oligopeptides
 - Cell wall associated proteinases** (Cell envelope proteinases = CEP)



LAB in cheeses (secondary flora/non-starter LAB)

- Spontaneously growing Non-Starter Lactic Acid Bacteria (NSLAB)
- Originate from the milk, ingredients used for cheese making or **the dairy environment**

•**Heterofermentative** lactobacilli:

- *Limosilactobacillus fermentum*
- *Levilactobacillus brevis*
- *Lentilactobacillus buchneri*
- *Lentilactobacillus parabuchneri*
- May cause cracks in cheese

•**Homofermentative**

- *Lactiplantibacillus plantarum*
 - *Lactiplantibacillus pentosus*
 - *Lacticaseibacillus casei*
 - *Lacticaseibacillus rhamnosus*
 - *Latilactobacillus curvatus*
 - *Loigobacillus bifermentans*
- *Pediococcus acidilactici*
 - *Pediococcus pentosaceus*
 - *Lactococcus laudensis*
 - *Lactococcus hircilactis*
 - *Lactococcus raffinolactis*

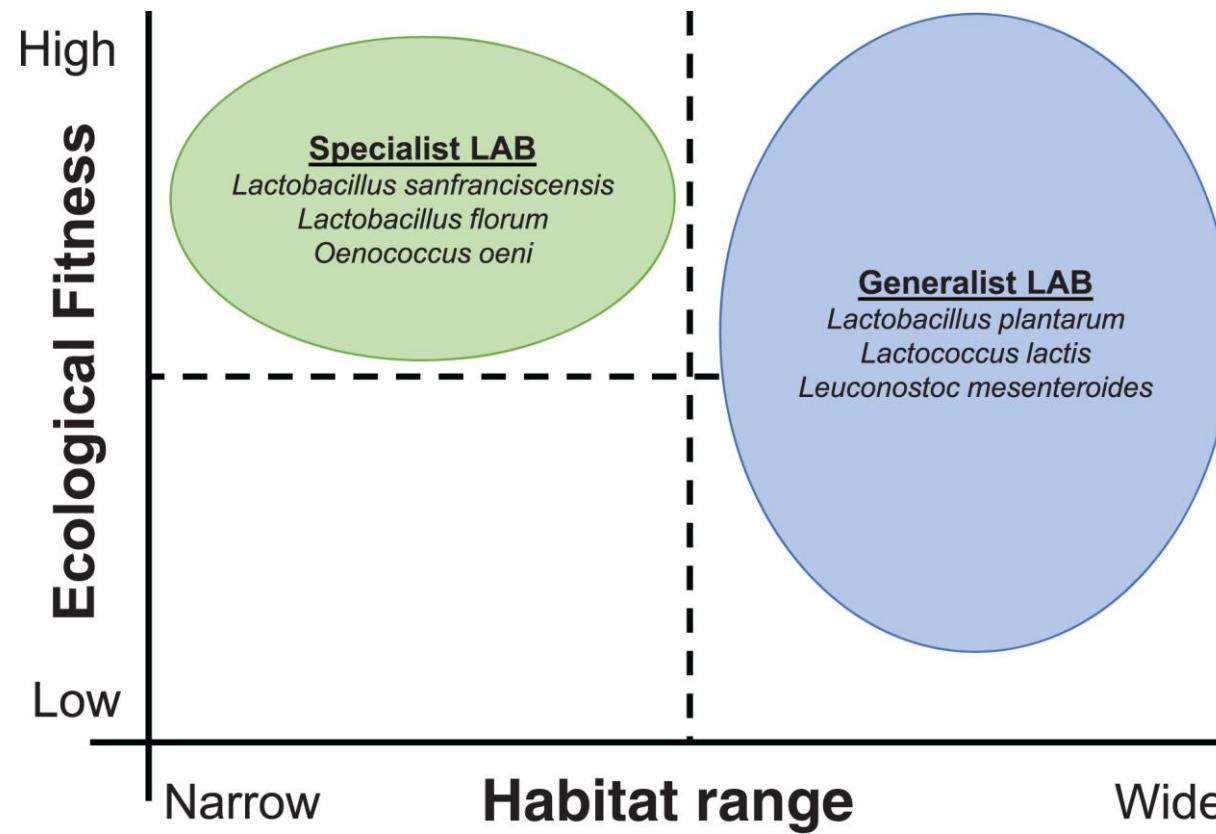
- May contribute to flavour development (both positive and negative)
- May control spoilage organisms and pathogens
 - Bacteriocin production
- May produce biogenic amines (associated with toxicity)
 - *L. brevis*, *L. buchneri* and *L. parabuchneri* strains reported positive for one or more
 - Histamine
 - Tyramine
 - Cadaverine
 - *L. casei* and pediococci strains reported positive for producing histamine



LAB and their roles in fermented foods...



LAB in plant fermentations



- Generalists often have larger genomes, with more accessory genes allowing growth in various environments
- Specialists are adapted to their niche and can outcompete others
- Many plant fermentations are dominated by generalist LAB
- Most common generalists isolated from plant material:
 - *Lactiplantibacillus plantarum*
 - *Lacticaseibacillus casei*
 - *Levilactobacillus brevis*
 - *Lactococcus lactis*
 - *Leuconostoc mesenteroides*
 - *Weisella cibaria*
- Composition modified by fermentation conditions (e.g. high salt, high nutrient, moderate temperature and low oxygen availability)

Role of LAB in sauerkraut

- Fermentation of carbohydrates into lactate, acetate, and sometimes CO₂
- Fermentation of organic acids present
 - Often a succession
 - *Leuconostoc* and *Weisella* initiate
 - Produce CO₂ and acids
 - Presence of acid promotes acid-tolerant *Levilactobacillus brevis* and also *Latilactobacillus curvatus*, *Latilactobacillus sakei*, *Enterococcus faecalis*, *L. lactis*
 - Sometimes *Pediococcus* and *L. plantarum* will end fermentation
 - Extensive CO₂ formation from heterofermentative LAB may cause spoilage



- Frequently a spontaneous fermentation dictated by:
 - Indigenous microflora
 - Temperature (15-20 ° C)
 - 2% of salt added
- Industrially more controlled by backslopping or use of starter cultures



LAB and their roles in fermented foods...



LAB in fermented cereal foods (sourdough)

- Homofermentative
 - *Companilactobacillus farciminis*
 - *Companilactobacillus crustorum*
 - *Companilactobacillus nantensis*
 - *Companilactobacillus alimentarius*
 - ***Lactiplantibacillus plantarum***
 - *Companilactobacillus mindensis*
 - *Latilactobacillus curvatus*
- Spontaneous, back-slopping (sourdough starters)
- Co-fermentation with yeasts
- Developed from indigenous microflora of the flour (for a few weeks of feeding before use to outcompete contaminants)
- Heterofermentative
 - ***Fructilactobacillus sanfranciscensis***
 - *Furfurilactobacillus rossiae*
 - *Furfurilactobacillus siliginis*
 - *Levilactobacillus acidifarinae*
 - ***Levilactobacillus brevis***
 - *Levilactobacillus namurensis*
 - *Levilactobacillus spicheri*
 - *Levilactobacillus zymae*
 - ***Limosilactobacillus fermentum***
 - *Limosilactobacillus frumenti*
 - *Limosilactobacillus pontis*
 - *Limosilactobacillus panis*
 - *Limosilactobacillus reuteri*
 - *Limosilactobacillus secaliphilus*
 - And probably more to come.



Role of LAB in sourdough fermentation

- Fermentation of low molecular weight carbohydrates (maltose, glucose, fructose and sucrose)
 - Production of lactic acid, acetic acid, ethanol and CO₂
- Degradation of starch, pectins, xylans and/or maltodextrins
 - Increased availability of carbohydrates (for us)
 - Prevention of staling
- Exopolysaccharide production (texture and prevention of staling)
- Proteolysis of grain proteins (flavour formation)
 - Availability of amino acids
 - Catabolism of amino acids
- Production of inhibitory compounds
 - Against spoilage bacteria (*Bacillus* spores)
 - Against molds (acids and other metabolites)
- Phytase activity
 - Degradation of phytate → Increased availability of minerals



LAB and their roles in fermented foods...



LAB in fermentation of meat (salami)

- *Companilactobacillus farciminis*
- *Companilactobacillus alimentarius*
- ***Lactiplantibacillus plantarum***
- *Lactiplantibacillus pentosus*
- ***Latilactobacillus sakei***
- ***Latilactobacillus curvatus***
- *Pediococcus pentosaceus*
- *Pediococcus acidilactici*



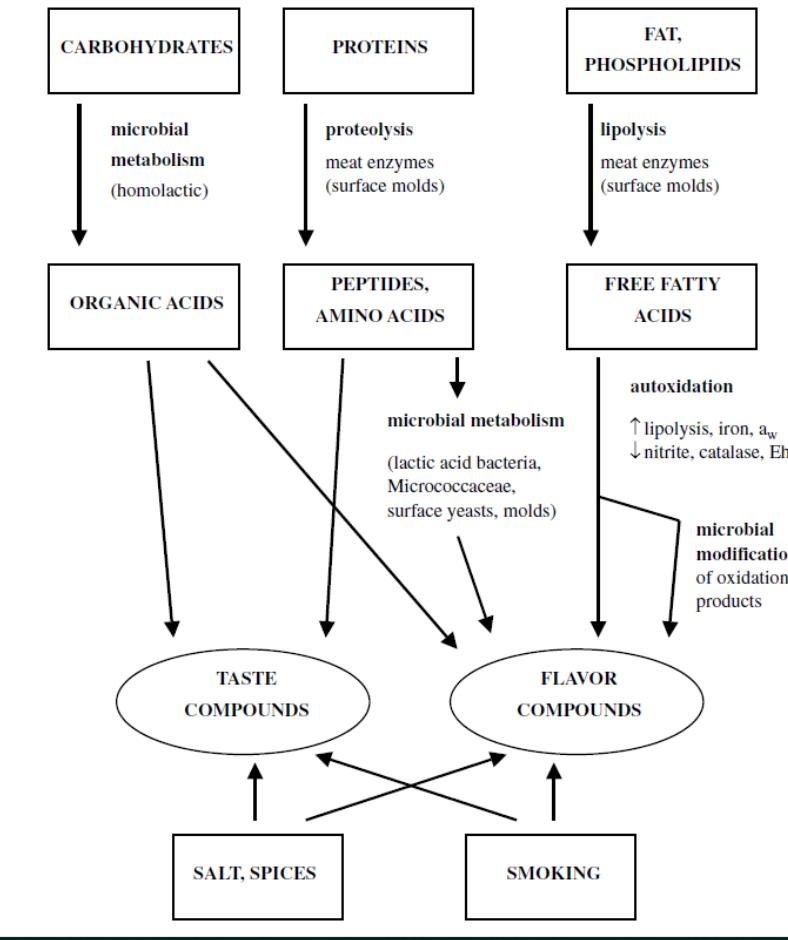
N.B. all homofermentative!
Heterofermentative LAB cause
spoilage due to CO₂ production

- Fermentation temperature and the sugars added (glucose, sucrose, lactose, maltodextrin, starch etc.) will determine which LAB are present
 - Starter cultures may be dominated by raw meat LAB microflora (and coagulase-negative staphylococci)



Role of LAB in sausage fermentation

- Fermentation of meat carbohydrates (and added carbohydrates) to lactic acid
 - Improved drying process (water loss due to low pH)
- Pseudocatalase activity will increase red colour development (e.g. *Latilactobacillus sakei*)
- Development of flavour
- Often made with starter culture and addition of nitrite/nitrate, salt and spices
- Artisanal fermentations often rely on indigenous microflora and salt/spices alone



LAB in meat spoilage (non-fermented meat products)

- LAB grow well in modified atmosphere packaging
 - Some also grow well at low temperature
 - *Carnobacterium*
 - *Leuconostoc*
- Spoilage is due to:
 - Acid formation
 - Gas production
 - **Exopolysaccharide formation (biofilm, slime)**
 - Biogenic amine production
 - Colour changes
 - H_2O_2
 - Green colour
- *Leuconostoc gelidum*
- *Leuconostoc carnosum*
- *Leuconostoc gasicomitatum*
- *Carnobacterium divergens* (homoferm)
- *Carnobacterium maltaromaticum* (homoferm)
- *Latilactobacillus sakei* (homoferm)
- *Latilactobacillus curvatus* (homoferm)
- *Weissella halotolerans*
- *Weissella viridescens*

Chemical state of myoglobin -- Ferric or Fe+++ (ionic bonds)

Compound	Color	Name
-CN	Red	Cyanmetmyoglobin
-OH	Brown	Metmyoglobin
-SH	Green	Sulfmyoglobin
- H_2O_2	Green	Choleglobin



LAB and their roles in fermented foods...



LAB in wine, cider, beer etc.

- Wine, sake & cider:

- ***Oenococcus oeni***
- *Liquorilactobacillus mali* (homoferm)
- *Companilactobacillus sakei* (homoferm)
- ***Lactiplantibacillus plantarum*** (homoferm)
- *Lentilactobacillus hilgardii*
- *Fructilactobacillus fructivorans*
- *Pediococcus parvulus* (homoferm)
- *Pediococcus inopinatus* (homoferm)

- Beer:

- *Pediococcus damnosus* (homoferm)
- *Pediococcus inopinatus* (homoferm)
- *Pediococcus claussenii* (homoferm)
- *Lactiplantibacillus plantarum* (homoferm)
- *Secundilactobacillus malefermentans*
- *Lentilactobacillus parabuchneri*
- *Levilactobacillus brevis*
- *Lentilactobacillus hilgardii*

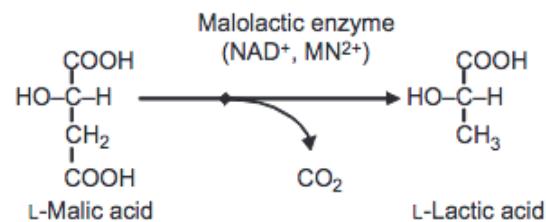
Used as starter culture or spontaneous fermentation by microbes from raw materials

For those interested, a nice review on LAB in wine: Virdis, C., Sumby, K., Bartowsky, E., & Jiranek, V. (2021). Lactic acid bacteria in wine: Technological advances and evaluation of their functional role. *Frontiers in Microbiology*, 11, 612118.



LAB as beneficial organisms in beverages

- Malolactic fermentation (wine)
 - *Oenococcus oeni*
 - *Lactiplantibacillus plantarum*
 - Used as starter cultures for wine



- In some beer types (sour beer, lambic, Berliner Weisse) LAB fermentation is wanted
 - Commercial cultures of *Levilactobacillus brevis*, *Lentilactobacillus hilgardii*, *Lactiplantibacillus plantarum*, *Pediococcus damnosus*
 - Acid production for acidic flavor
 - Other not well-defined aroma compounds produced

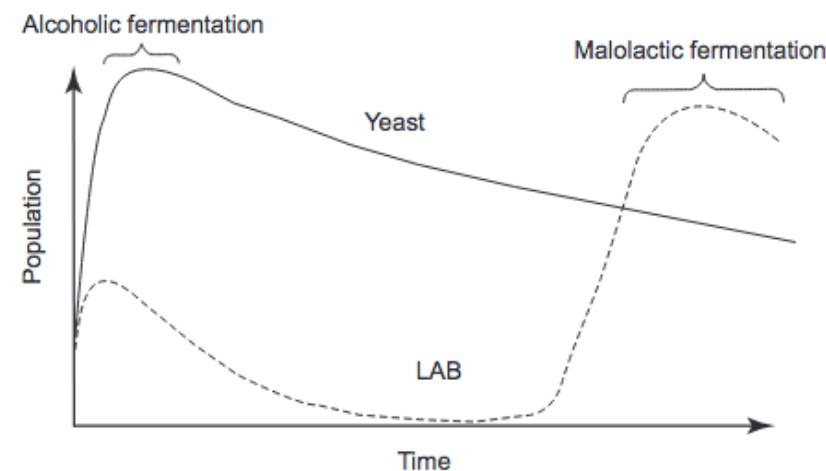
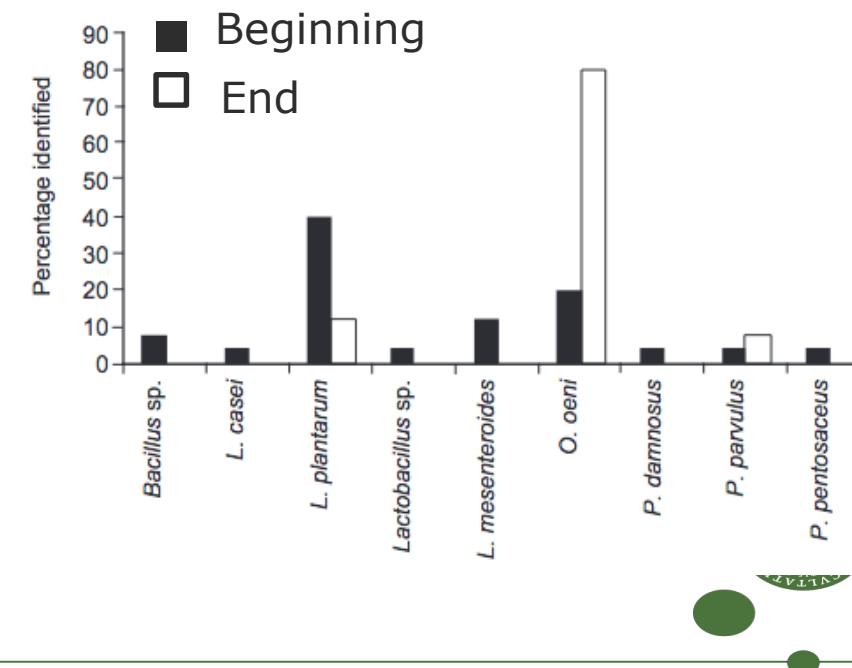


Fig. 3.1 General evolution of yeast and lactic acid bacteria populations during winemaking.



LAB in spoilage of beverages

- Pyruvate fermentation may produce diacetyl
 - Unpleasant in fat-free environments
 - Diacetyl may also be produced by the yeast from carbohydrates
- Acid formation from sugars may be unwanted
 - In beer, hops normally control LAB microflora
 - LAB with hop resistance may spoil beer
- Polysaccharide formation may produce cloudy beer that can not be filtered
- Undesirable aroma compounds produced from amino acid catabolism



Take home messages

- LAB are involved in fermentations of a diverse range of foods from different raw materials
 - Vegetables, cereals, roots and tubers, milk, meat, fruit (see Tamang, J. P., Watanabe, K. and Holzapfel, W.H. (2016) Review: Diversity of Microorganisms in Global Fermented Foods and Beverages. *Front. Microbiol.* 24, 377 <https://doi: 10.3389/fmicb.2016.00377> for more details)
- LAB appear as indigenous flora, through back-slopping or as starter cultures depending on the fermentation process used for the fermented food
- Many roles in fermentations related to species and metabolism
 - Especially acidification, CO₂ production and flavour formation
 - Inhibition of other microorganisms
- In most fermented foods LAB do not perform the fermentation alone but together with yeasts, moulds and/or other bacteria



Common Fermentation Organisms in Food

Common Fermentation Organisms in Food									
Lactobacillaceae		Streptococcaceae, Enterococcaceae		Other bacteria		Fungi		Yeasts	
Lactobacillus	L.	Lactococcus	Lc.	Acetobacter	Ac.	Aspergillus	A.	Saccharomyces	S.
Leuconostoc	Lu.	Streptococcus	Sc.	Staphylococcus	St.	Penicillium	P.	Candida	C.
Weissella	W.	Tetragenococcus	T.	Gluconacetobacter	Gl.	Geotrichum	G.	Debaromyces	D.
Pediococcus	Pc.	Enterococcus	E.	Bacillus; Lentibacillus	Bc.; Lt	Monascus	M.	Kluyveromyces	K.
Oenococcus	O.	Non-starter lactic acid bacteria	NSLAB	Brevibacterium	Br.	Rhizopus	R.	Zygosaccharomyces	Z.
				Propionibacterium	Pr.	Botrytis	B.	Blastobotrys	Bl.

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, 2, 106-117.

Current Opinion in Food Science

Periodic table of fermented foods:
diversity of products,
fermentation organisms,
and raw materials.

Fermented foods are grouped by product category and ranked within a group by flavour intensity or ripening time where applicable.

Colour coding of specific fields indicates the presence of specific groups of fermentation organisms (see key on top of table); typical organisms, typical concentration of metabolites, and characteristic ripening/fermentation times are indicated.

Water activities are not presented for dry foods or alcoholic beverages.





Faculty of Science

Acetic Acid Bacteria

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MFFB AAB
Dias 1



Acetic acid bacteria

Acetic Acid Bacteria (AAB)

- 19 genera and more than 90 species recognised
- Ellipsoid to rod-shaped
- Gram negative (Gram variable in a few cases)
- Oxygen required for growth
- Catalase positive
- Oxidase negative
- pH optimum 5-6.5
- Mesophilic, optimal growth around 25-30 ° C
 - Some strains grow at higher temp.
- Some species able to grow down to pH ~ 3
- Oxidise sugars and/or alcohols to organic acids
 - Ethanol + O₂ -> Acetic acid + H₂O
 - *Asaia* spp. the only exception. Does not oxidise ethanol to acetic acid



Classification

- Phylum: Proteobacteria
- Class: Alpha Proteobacteria
- Family: Acetobacteraceae
- Genera:

Table 1

Presently described genera of the acetic acid bacteria with the type species of the genus and the number of described species.

Genus	Type species	Number of described species	Author(s)
<i>Acetobacter</i>	<i>A. aceti</i>	25	Beijerinck (1898)
<i>Gluconobacter</i>	<i>G. oxydans</i>	15	Asai (1935)
<i>Acidomonas</i>	<i>A. methanolica</i>	1	Ura Kami et al. (1989)
<i>Gluconacetobacter</i>	<i>G. liquefaciens</i>	10	Yamada et al. (1997)
<i>Asaia</i>	<i>A. bogorensis</i>	8	Yamada et al. (2000)
<i>Kozakia</i>	<i>K. baliensis</i>	1	Lisdiyanti et al. (2002)
<i>Saccharibacter</i>	<i>S. floricola</i>	1	Jojima et al. (2004)
<i>Swaminathania</i>	<i>S. salitolerans</i>	1	Loganathan and Nair (2004)
<i>Neasacia</i>	<i>N. chiangmaiensis</i>	1	Yukphan et al. (2005)
<i>Granulibacter</i>	<i>G. bethesdensis</i>	1	Greenberg et al. (2006)
<i>Tantcharoenia</i>	<i>T. sakaeratensis</i>	1	Yukphan et al. (2008)
<i>Commensalibacter</i>	<i>C. intestinali</i>	1	Roh et al. (2008)
<i>Ameyamaea</i>	<i>A. chiangmaiensis</i>	1	Yukphan et al. (2009)
<i>Neokomagataeza</i>	<i>N. thailandica</i>	2	Yukphan et al. (2011)
<i>Komagataeibacter</i>	<i>K. xylinus</i>	14	Yamada et al. (2012a,b)
<i>Endobacter</i>	<i>E. medicaginis</i>	1	Ramirez-Bahena et al. (2013)
<i>Swingsia</i>	<i>S. samuiensis</i>	1	Malimas et al. (2013)
<i>Nguyenibacter</i>	<i>N. vanlangensis</i>	1	Thi Lan Vu et al. (2013)
<i>Bombella</i>	<i>B. intestinali</i>	1	Li et al. (in press)

Every year since 1998 (with the exception of 2003) at least one new AAB species has been described.

Always consult
<https://lpsn.dsmz.de/>
 for an updated list of
 recognised species

Good and quite recent (2021)
 review on AAB classification:

Qiu et al. AMB Expr. (2021) 11:29
<https://doi.org/10.1186/s13568-021-01189-6>

AMB Express

MINI-REVIEW

Open Access

Classification of acetic acid bacteria and their acid resistant mechanism

Xiaoman Qiu^{1,2}, Yao Zhang^{1,2} and Housheng Hong^{1,2*}



Phenotypic differentiation

Table 2
Differential characteristics of the twelve genera of acetic acid bacteria.

Characteristic	A	Ac	As	Ga	G	K	S	Sa	N	Gr	T	Am
Flagellation	pe/n	n	pe/n	pe/n	po/n	n	pe	n	n	n	n	po
Oxidation of ethanol to acetic acid	+	+	-/M ^a	+	+	+	+	v	+	v	+	+
Oxidation of acetic acid to CO ₂ and H ₂ O	+	+	+	+	-	w	w	-	-	w	-	+
Oxidation of lactate to CO ₂ and H ₂ O	+	w	+	+/-	-	w	w	w	-	+	-	w
Growth on 0.35% acetic acid-containing medium	+	+	-	+	+	+	+	-	+	Nd	+	+
Growth on methanol	-/W ^c	+	-	-	-	-	-	-	-	+	-	w
Growth on D-mannitol	+/-	w	+/-	+/-	+	+	+	-	-	-	-	-
Growth in the presence of 30% D-glucose	-	-	+	+/-	-/+	-	Nd	+	+	Nd	+	-
Production of cellulose	-	-	-	+/-	-	-	Nd	-	Nd	Nd	-	-
Production of levan-like mucous substance from sucrose	-/+	-	-	-/+	-	+	Nd	-	-	Nd	-	-
Fixation of molecular nitrogen	-	-	-	-/+	-	-	-	-	-	-	-	-
Ketogenesis (dihydroxyacetone) from glycerol	+/-	w	-/w	+/-	+	+	+	-	w	-	+	w
<i>Acid production from:</i>												
D-Mannitol	-/+	-	+/-	+/-	+	-	-	+	w	-	-	-
Glycerol	-/+	-	+	+	+	+	+	-	+	v	+	w
Raffinose	-	Nd	-	-	+	Nd	-	+	+	Nd	-	-
Cellular fatty acid type	C _{18:1}	-	-	-	-	-	-					
Ubiquinone type	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
DNA base composition (mol% G + C)	52–60	63–66	59–61	55–66	55–63	56–57	57–60	52–53	63.1	59	66.0	65.6

Abbreviations: A, *Acetobacter*; Ac, *Acidomonas*; As, *Asaia*; Ga, *Gluconacetobacter*; G, *Gluconobacter*; K, *Kozakia*; S, *Swaminathania*; Sa, *Saccharibacter*; N, *Neoasaia*; Gr, *Granulibacter*; T, *Tanticharoenia*; Am, *Ameyamaea*; pe, peritrichous; po, polar; n, none; +, 90% or more of the strains positive; w, weakly positive reaction; -, 90% or more of the strains negative; v, variable; Nd, not determined.

^a *Asaia* does not produce acetic acid from ethanol with the exception of one strain producing acid weakly (Yamada et al., 2000).

^b Overoxidation of acetate to CO₂ and H₂O depends on acetate concentration in medium.

^c *A. pomorum* assimilates methanol weakly (Sokollek et al., 1998b).

Table is adapted from Sievers and Swings (2005) with combining data from Loganathan and Nair (2004) (for *Swaminathania*); Jojima et al. (2004) (for *Saccharibacter*); Yukphan et al. (2005) (for *Neoasaia*); Greenberg et al. (2006) (for *Granulibacter*) Yukphan et al. (2008) (for *Tanticharoenia*); Yukphan et al. (2009) (for *Ameyamaea*).

- Species differentiation: Consult Bergeys Manual, part 3, The Proteobacteria and International Journal of Systematic and Evolutionary Microbiology and/or:



AAB identification

- AAB identification based on phenotypic tests tedious and difficult
- Molecular biology based tests often used
- PCR-RFLP-analysis
- Rep-PCR, often $(GTG)_5$ -based
- 16S rRNA gene and/or 16S-23S rRNA gene internal transcribed spacer sequencing
- MALDI-TOF
- Multi-locus sequence analysis (MLSA)
- Full genome sequencing



16S rRNA gene sequencing/Family relatedness

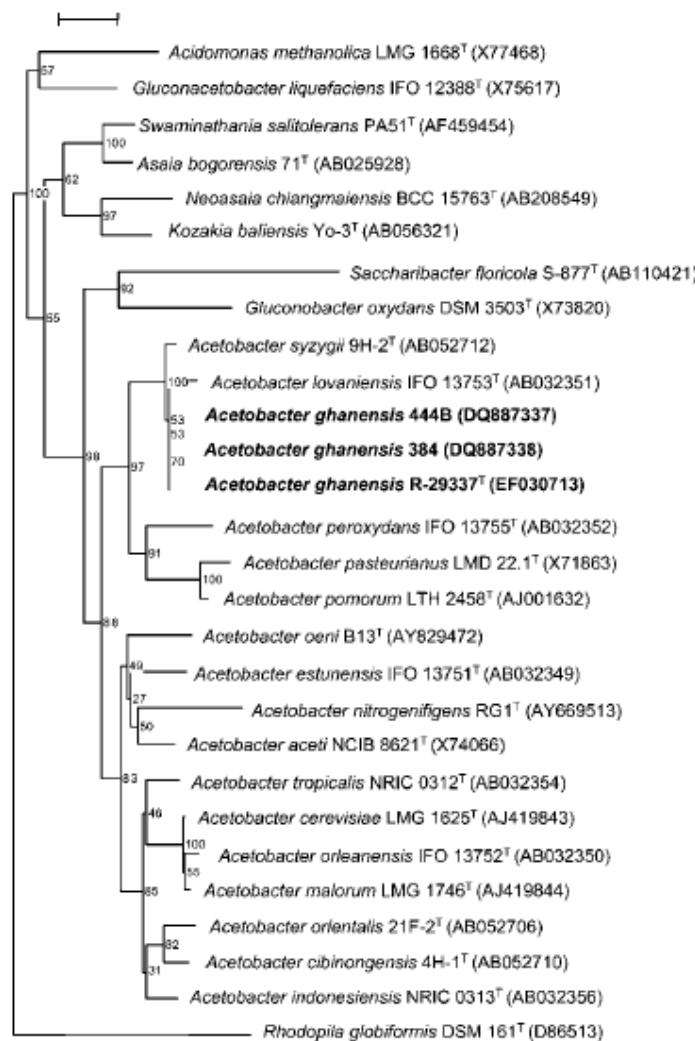


Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter ghanensis* sp. nov. and related species of the family Acetobacteraceae. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

Cleenwerck et al. (2007).
IJSEM, 57:1647-1652



DNA:DNA hybridisation

Table 2. DNA-DNA binding values and G+C content of *Acetobacter* strains studied

Mean standard deviation of DNA-DNA hybridization is $\pm 7\%$ (see Goris *et al.*, 1998).

Strain	G+C content (mol%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34							
<i>A. pasteurianus</i>																																										
1. LMG 1555	54.3		100																																							
2. LMG 1686	53.5			100																																						
3. LMG 1262 ^T t1	53.4	78	82	100																																						
4. LMG 1630	53.7	79	71	100																																						
5. LMG 1629	53.6	90	70	100																																						
6. LMG 1658	53.2	66	62	59	100																																					
7. LMG 1659	53.7	69	66	100																																						
<i>A. pomorum</i>																																										
8. LMG 18848 ^T	52.1	51	58	53	51	55		55	100																																	
<i>A. peroxydans</i>																																										
9. LMG 1633	60.7							6				9	100																													
10. LMG 1635 ^T	59.7							13				23	73	100																												
<i>A. lovaniensis</i>																																										
11. LMG 1617 ^T	57.4		21	14		18			20	17	16	100																														
<i>A. estunensis</i>																																										
12. LMG 1626 ^T	59.2		20					13	9		10	100																														
13. LMG 1572	60.2	19	21	21	19	3		12	7		9	98	100																													
14. LMG 1580	59.5							92	92	100																																
<i>A. aceti</i>																																										
15. LMG 1531	58.3		16	18		6		12			16																															
16. LMG 1535	57.0		14	10		8		8			14																															
17. LMG 1504 ^T	56.9	7	8	7	9	6	5	6	6	10	13	17	17																													
18. LMG 1496	56.9		6	9		7		7			12																															
<i>A. cerevisiae</i> sp. nov.																																										
19. LMG 1625 ^T	57.6		37	32		19		27	10		11	15	17		18	16	12	15	100																							
20. LMG 1599	56.8			30				23			25								11	77	100																					
21. LMG 1699	56.0			35				24			27								8	74	76	100																				
22. LMG 1682	57.4		22	32		13		19			18	15			18	10	9	9	85	80	66	100																				
<i>A. malorum</i> sp. nov.																																										
23. LMG 1746 ^T	57.2	10	11	7	8	6		9	7		9	5	5						3	53	50		100																			
<i>A. orleanensis</i>																																										
24. LMG 1583 ^T	55.7					12			12	11		10	6	7					4	33	37	37	40	28	100																	
25. LMG 1592	58.0*																																									
26. LMG 1608	58.1*																																									
27. LMG 1545	57.3*																																									
<i>A. indonesiensis</i>																																										
28. LMG 19824 ^T	54.0																																									
29. LMG 1588	54.2			17				16	7		15	13	18						9	20	26	32	20	10	12																	
30. LMG 1571	54.1																																									
<i>A. tropicalis</i>																																										
31. LMG 19825 ^T	56.0																																									
32. LMG 19826	55.6																																									
33. LMG 1754	56.2		18		11	12												10	10	9	8	27																				
34. LMG 1663	55.9	22	20	14	6	14	10	6	14	13	13	10	10	9	8	27																										

* Data taken from Lisdiyanti *et al.* (2000).



PCR-RFLP

- Often 16S rRNA gene-based =>
- Amplify 16S rRNA gene (or other gene) by PCR
- Cut with restriction enzyme(s)

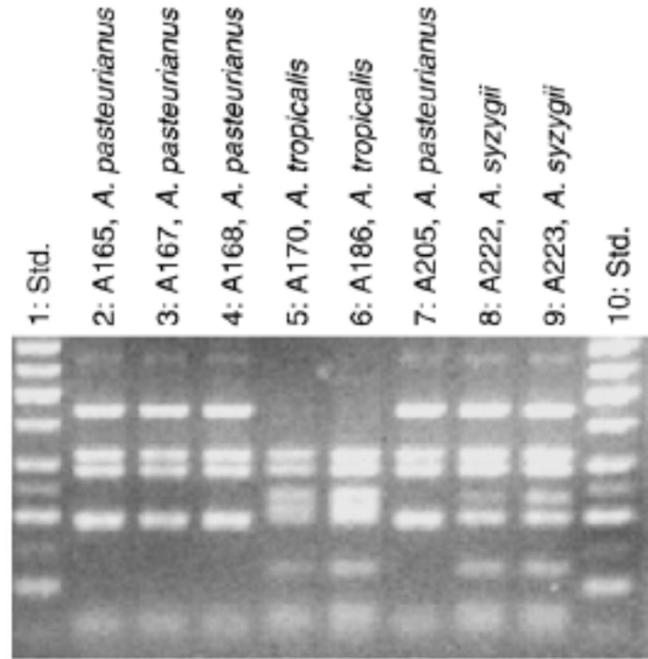


Fig. 3. Typical profile of *Alu*I digested 16S rRNA gene fragments of Acetic Acid Bacteria isolated during cocoa fermentations. A 50 bp molecular size marker was used a standard. Abbreviations: *A.*: *Acetobacter*, Std.: Standard.



PCR-RFLP-based identification of AAB

Table 3

Restriction fragment sizes of the amplified 16S DNA used to classify the AAB

<i>Aha</i> I	<i>Cfo</i> I
A1	330–280–250–210–200–120–50
A2	450–320–290–200–120–50
A3	450–320–290–200–110–50
A4	550–290–210–190–190
A5	550–290–200–180–120–70–50
A6	790–490–120–50
A7	750–290–210–180–90
A8	750–280–210–120–70
A9	740–470–120–70–50
A10	550–275–225–210–190
A11	550–250–225–225–175
<i>Tru</i> 9I	
Tr1	530–350–350–150–110
Tr2	530–350–340–150–110
<i>Taq</i> I	
T1	650–375–210–180
T2	500–375–370–210
T3	850–375–210
T4	500–375–210–175–160
T5	500–375–200–190–150
ITS	
ITSA1	480–210–120–50
ITSA2	480–280–110
ITSC1	400–210–160–70
ITSC2	500–190–160
ITSP1	250–500 ^a
ITSP2	300–500 ^a

^a Data obtained from Sievers et al. (1996).



PCR-RFLP-based identification of AAB

Table 2

Classification of the AAB according to the electrophoretic analysis of the PCR-RFLP of 16S rDNA and RFLP-PCR of ITS 16S–23S rDNA

		<i>Alu</i> I	<i>Cfo</i> I	<i>Tru</i> 9I	<i>Hinf</i> I	<i>Taq</i> I	<i>Hae</i> III	<i>Rsa</i> I	ITS+ <i>Alu</i> I	ITS+ <i>Cfo</i> I	ITS+ <i>Pvu</i> II
1	<i>A. tropicalis</i>	A1	C1	Tr1		T1					
2	<i>A. indonesiensis</i>		C1	Tr1		T3					
3	<i>A. cerevisiae</i>		C1	Tr2					ITSA1	ITSC2	
4	<i>A. orleanensis</i>		C1	Tr2					ITSA2		
5	<i>A. malorum</i>		C1	Tr2					ITSA1	ITSC1	
6	<i>A. estuensis</i>		C2		Hil						
7	<i>A. aceti</i>		C2		H2						
8	<i>A. orientalis</i>	A2				T1					
9	<i>A. cabinongensis</i>					T3					
10	<i>A. syngii</i>	A3				T4					
11	<i>A. lovaniensis</i>					T5					
12	<i>A. pasteurianus</i>					T2					
13	<i>A. pomorum</i>					T2					
14	<i>K. baliensis</i>	A4									
15a	<i>G. frateurii</i>	A5	C1								
15	<i>G. cerinus</i>		C3								
16	<i>G. oxydans</i>		C4								
17	<i>Ga. intermedium</i>	A6				H1					
18	<i>Ga. xylinus</i>					H4				ITSP1	
19	<i>Ga. europaeus</i>					H4				ITSP2	
20	<i>Ga. johannae</i>	A7					R2				
21	<i>Ga. azotocaptans</i>						R1				
22	<i>Ga. sacchari</i>	A8				H3					
22b	<i>Ga. diazotrophicus</i>					H2	R1				
23	<i>Ga. liquefaciens</i>					H2	R2				
24	<i>Ga. oboediens</i>	A9			H2						
25	<i>Ga. hansenii</i>				H3						
26	<i>Ac. methanolica</i>	A10									
27	<i>As. bogorensis</i>	A11									

ITS+restriction endonuclease means the use of PCR-RFLP of the 16S–23S ITS rDNA.



(GTG)₅-based fingerprinting of AAB

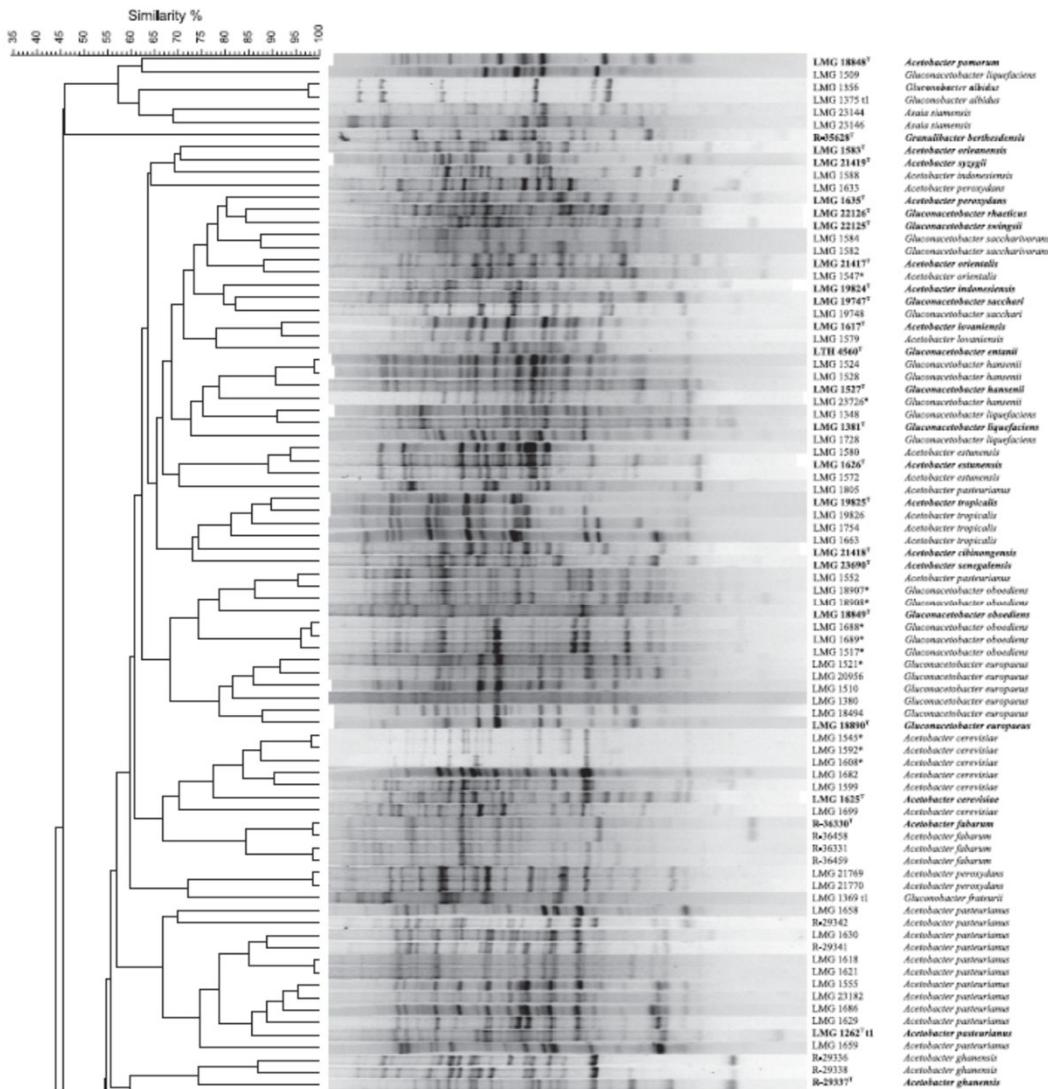


Fig. 1. (GTG)₅-PCR banding patterns of 158 strains of AAB, including *Acetobacter* species (68 strains), *Ascia* species (11 strains), *Acidomonas* species (4 strains), *Gluconacetobacter* species (45 strains), *Gluconobacter* species (25 strains), and other AAB, namely *Granulibacter bethesdensis* R-35628^T, *Kozakia baliensis* LMG 21812^T, *Neoasia chiangmaiensis* LMG 24037^T, *Saccharibacter floricola* LMG 23170^T, and *Swaminathania salitolentans* LMG 21291^T. Type strains are in bold. *Strains suggested for reclassification. The dendrogram was generated after cluster analysis of the digitized fingerprints and was derived from UPGMA linkage of Pearson correlation coefficients.



16S rRNA gene sequencing

- Sanger sequencing followed by Blast-search

```
>gb|DQ523494.1| Acetobacter tropicalis strain A77 16S ribosomal RNA gene, partial sequence
Length=1419

Score = 2560 bits (2838), Expect = 0.0
Identities = 1419/1419 (100%), Gaps = 0/1419 (0%)
Strand=Plus/Plus

Query   1      TGATCATGGCTCAGAGCGAACGCTGGCGGCATGCTAACACATGCAAGTCGCACGAAGGT    60
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct   1      TGATCATGGCTCAGAGCGAACGCTGGCGGCATGCTAACACATGCAAGTCGCACGAAGGT    60
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Query   61     TTCGGCCTTAGTGGCGGACGGGTGAGTAACCGTAGGAATCTATCCATGGGTGGGGATA    120
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct   61     TTCGGCCTTAGTGGCGGACGGGTGAGTAACCGTAGGAATCTATCCATGGGTGGGGATA    120
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Query   121    ACTCTGGAAACTGGAGCTAATACCGCATGATACCTGAGGGTCAAAGGCGCAAGTCGCCT    180
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct   121    ACTCTGGAAACTGGAGCTAATACCGCATGATACCTGAGGGTCAAAGGCGCAAGTCGCCT    180
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Query   181     GTGGAGGAGCCTGCGTTGATTAGCTTGGTGGGTAAATGGCCTACCAAGGCGATGAT    240
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct   181     GTGGAGGAGCCTGCGTTGATTAGCTTGGTGGGTAAATGGCCTACCAAGGCGATGAT    240
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Query   241     CGATAGCTGGTCTGAGAGGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT    300
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct   241     CGATAGCTGGTCTGAGAGGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT    300
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Query   301     ACGGGAGGCAGCAGTGGGAATATTGGACAATGGGGCAACCCTGATCCAGCAATGCCGC    360
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
```



MultiLocus Sequence Analysis

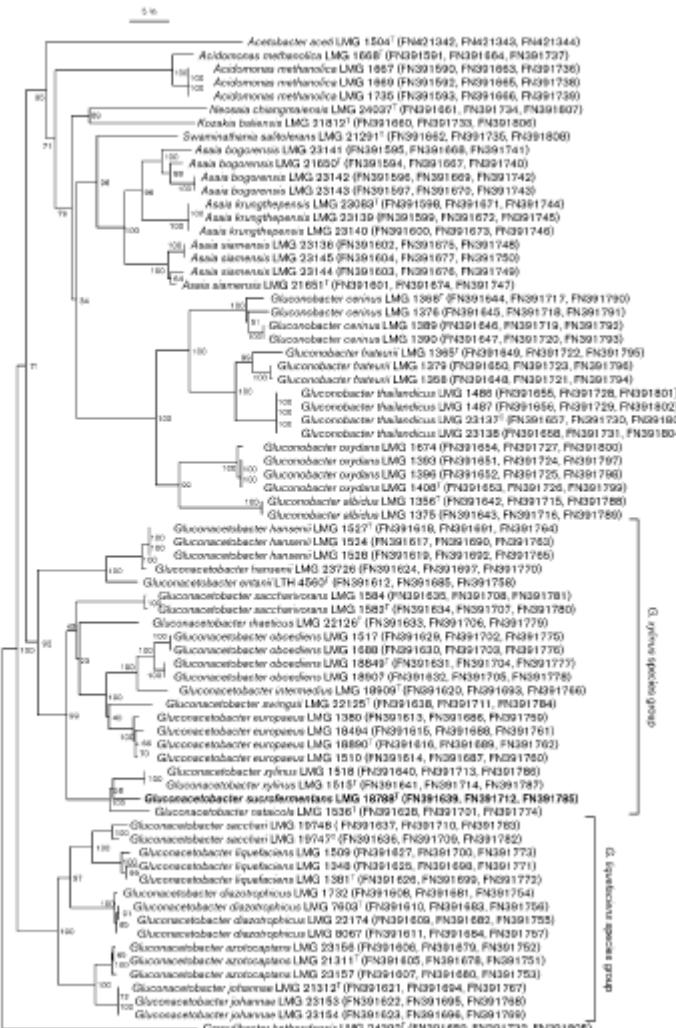
Table 1. Amplification and sequencing primers used in this study

Primer positions are given relative to the genome sequence of *G. oxydans* 621H.

Primer	Sequence (5'-3')	Position
dnaK-01-F	CTGGGCATCATCAACGAGCC	925719–925738
dnaK-02-R	CTCACGCTGCCCTGATAGA	926546–926527
dnaK-03-F	TTCGACGTSTCCATCCTCGA	925818–825837
dnaK-04-R	TTGGTCGGGATCGTCGTGTT	926470–925451
groEL-10-F	ACAAGTTGGAGAACATGGGC	2083114–2083133
groEL-11-R	TCCTTGGCTCCCTCACCTC	2084104–2084085
groEL-12-F	TSAAGCGGGCATCGACAA	2083270–2083288
groEL-13-F	CTGATCCACGAAAAGAACGT	2083584–2083603
groEL-14-R	TGCAACGGGCTTGTATGTC	2083962–2083944
groEL-15-R	AGCTTCTTTCTGGATCATG	2083603–2083584
rpoB-01-F	GATAACGGCACCTICATCAT	405644–405625
rpoB-02-R	AGATTGTCGATATCGTCGAT	404620–404639
rpoB-03-F	GATCACGACAAGGGCAAGAC	405566–405547
rpoB-04-F	CCAAGCTGACCGCSCGTA	405157–405140
rpoB-05-R	ATGTTICATCITCACACGACCG	404743–404763
rpoB-06-R	TACGGCGGTCAAGCTTGG	405140–405157
rpoB-07-R	AGRCCGATGTTCATCTTSA	404737–404755
recA_A_F	AAGGCTCGATCATGCGCATG	1676846–1676827
recA_A_R	AYCTTGCTGATGCCCTCWCC	1676104–1676121
thrC_A_F	GAACTGITYCACGCC	International Journal of Systematic and Evolutionary Microbiology (2010), 60, 2277–2283
thrC_A_R	TCCAGCARITCGAA	
gltA_A_F	CTTCCGGCGCGATG	
gltA_A_R	ACAGCACSGTGAAC	

DOI 10.1099/ijss.0.018465-0

Cultivate/propagate
Extract DNA
Amplify (PCR)
Sequence
Analyse



AAB cultivation

- Cultivation of AAB is quite difficult (!!)
- They often occur in environments where especially LAB and yeast also thrive
- Your tasks:
 - **Propose a medium** (solid substrate) that selects for AAB *and* contain some sort of “indicative principle” (some sort of feature that indicate to you, that a given colony might be an AAB)
 - **Suggest** a couple of easy and fast **phenotypic tests**, that allows you to discriminate between AAB, LAB and yeast in a few minutes
 - Work in groups of 3-4. **You have 7 minutes**



AAB cultivation and long term storage

Table 3–Media commonly used for the growth of AAB and their composition.

Ingredient (%)	GYC	AE	YPM	MYA	DMS	mDMS
Glucose	10	0.5	-	-	0.1	0.1
Yeast extract	1	0.3	0.5	0.5	0.3	0.3
Peptone	-	0.4	0.3	-	1.0	1.0
Calcium carbonate	2	-	-	-	-	-
Ethanol	-	3	-	6	-	0.5
Glacial acetic acid	-	3	-	-	-	0.3
Lactic acid	-	-	-	-	-	0.6
Mannitol	-	-	2.5	-	0.1	0.1
Sorbitol	-	-	-	-	0.1	0.1
Malt extract	-	-	-	1.5	-	-
Calcium lactate	-	-	-	-	1.5	-
Potassium phosphate	-	-	-	-	0.1	0.1
Sodium deoxycholate	-	-	-	-	0.01	0.01
Magnesium sulfate	-	-	-	-	0.002	0.002
Bromocresol	-	-	-	-	0.003	0.003
Bacteriological agar	1.5	0.9	1.2	1.5	1.0	1.8

GYC, glucose-yeast extract-calcium carbonate; AE, acetic acid-ethanol; YPM, yeast extract-peptone-mannitol; MYA, malt extract-yeast extract-acetic acid; DMS, deoxycholate-mannitol-sorbitol; mDMS, modified deoxycholate-mannitol-sorbitol.

- Different substrates developed
- Incubation: 25-30 ° C, 3-6 days
- Some “environments” difficult
- Long term storage sometimes difficult
 - YG with 20% glycerol and storage at -80 ° C (normally) allow storage for years



Technological properties

- Alcohol oxidised to acids
- Ethanol + O₂ -> Acetic acid + H₂O
 - Exothermal process
- Some species over-oxidise acetic acid to CO₂
 - *Acetobacter* spp., *Acidomonas* spp., *Asaia* spp. and *Gluconacetobacter* spp.
- Some strains: Alcohols oxidised into sugars
 - E.g. mannitol into fructose
 - *Gluconobacter* industrially important, produce
 - L-Sorbose from D-sorbitol
 - D-gluconic acid, 5-keto- and 2-keto-D-gluconate from D-glucose
 - Di-hydroxyacetone from glycerol

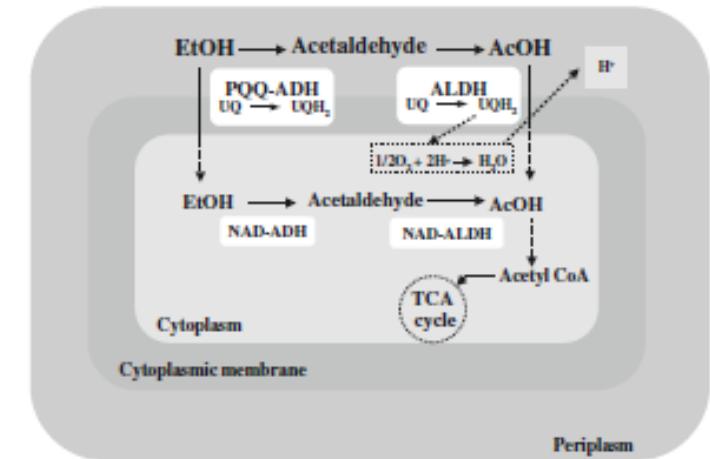
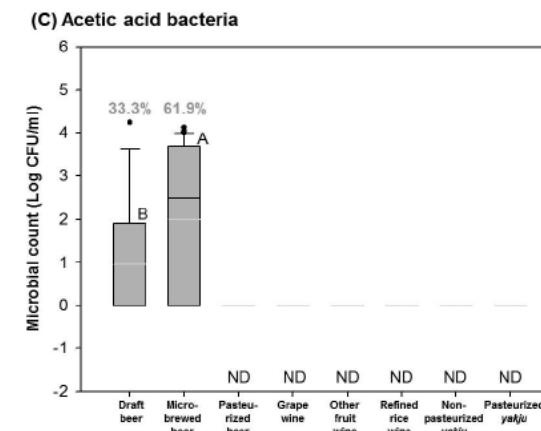


Fig. 1 Ethanol oxidation by PQQ-ADH and ALDH at the outer surface of cytoplasmic membrane and by NAD-ADH and NAD-ALDH in the cytoplasm



Technological properties

- *Acetobacter xylinum*
 - Produces cellulose from glucose
- Some species nitrogen-fixing
 - *Ga. diazotrophicus*, *Ga. Azotocaptans*, *Ga. Johannae*, *S. salitolerans*, *A. peroxydans* and *A. nitrogenifigens*
- But also spoilage organism in some cases
 - Where?
- Opportunistic pathogen?
 - *Asaia bogorensis* (and in rare cases *Acetobacter* and *Gluconobacter*) colonize catheters
 - *Granulibacter bethesdeniae* and *Acidomonas methanolica* isolated from patients with chronic granulomatous disease



AAB and fermented foods

- Vinegar
 - Generally *A. pasteurianus*, but also other species
 - Strain succession
 - Two methods, the “traditional” and the submerged process
- Coffee
 - Role unknown
- Cocoa
 - Microbial succession
- Lambic
- Kombucha
- Some sourdoughs
 - Exopolysaccharide production



Cocoa:

Table 2
Temperature of the fermenting mass, pulp pH and log(CFU/g) of yeasts, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and *Bacillus* spp. (standard deviations in brackets) determined 15 cm from the surface of a large heap cocoa fermentation turned after 48 and 96 h

	Fermentation time (h)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Temperature, °C	28	29.5	34	42	43	48	48	44	42	44.5	44	46	44
pH, pulp	4.10	4.50	4.24	3.93	3.98	4.03	4.21	4.37	4.40	4.35	4.49	4.58	4.55
Yeast, log(CFU _{yeast} /g)	6.97 (0.04)	7.24 (0.03)	6.60 (0.21)	4.90 (0.43)	5.38 (0.25)	5.08 (0)	6.87 (0.02)	6.67 (0.16)	7.32 (0.34)	5.40 (0.02)	7.86 (0.12)	5.65 (0.14)	5.55 (0.14)
% of yeast population													
<i>H. guilliermondii</i>	79	67	71										
<i>S. cerevisiae</i>	7												
<i>P. stipitata</i>	9	2											
Unidentified Species C	3												
<i>I. hanensis</i>	2												
<i>C. zemplinina</i>		19	14										
<i>C. michailovi</i>		3											
<i>C. diversa</i>		9	14										
<i>P. membranifaciens</i>				Det. ^a	100	70	50	100	100	90	100	100	100
<i>C. ethanolica</i>						15							
<i>Sc. cerevisiae</i>						15	17						
<i>Schiz. pombe</i>							33						
<i>I. orientalis</i>										10			
LAB, total log(CFU _{LAB} /g)	5.99 (0.12)	8.09 (0.04)	8.86 (0.15)	9.08 (0.11)	9.03 (0.10)	8.47 (0.02)	9.09 (0.27)	9.25 (0.03)	9.23 (0.12)	6.72 (0.03)	9.16 (0.06)	8.73 (0.07)	8.88 (0.03)
% of LAB population													
<i>Lb. plantarum</i>	60	11	5		9		9						
<i>Lb. fermentans</i>	40	81	76	100	86	100	81	89	92	69	21	85	63
Unidentified Species D	8							11	8		74	15	37
<i>Lc. pseudoliquescens</i>		19									5		
<i>Lb. kiligandii</i> ^b					5								
<i>Pd. acidilactici</i>										31			
AAB, log(CFU _{AAB} /g)	5.60 (0)	N.D. ^c	7.14 (0.02)	7.33 (0.17)	7.76 (0.02)	6.94 (0.01)	No data ^d	7.54 (0.06)	7.38 (0)	5.41 (0.05)	8.14 (0.09)	5.84 (0.34)	5.99 (0.12)
% of AAB population													
<i>A. pasteurianus</i>	100			55		22				62			
<i>A. xylophilus</i>			57			33		72					
<i>A. tropicalis</i>	29		45	100	45			14	100	38	100	100	100
<i>A. malorum</i>			14										
<i>G. oxidans</i>							14						
Bacillus spp. log(CFU _{Bacillus} /g)	N.D. ^c	N.D. ^c	N.D. ^c	N.D. ^c	5.49 (0.15)	7.74 (0.09)	N.D. ^c	3.60 (0)	9.10 (0.71)	5.75 (0.21)	7.99 (0.12)	7.75 (0.04)	7.81 (0.19)
% of Bacillus population													
<i>B. licheniformis</i>					100				50	100	72	96	80
<i>B. cereus</i>						Det. ^a							
<i>B. pumilus</i>							100		100	50		18	4
<i>B. megaterium</i>											10		11
<i>B. subtilis</i>												9	

Abbreviations: *H.*: *Hanseniaspora*, *S.*: *Saccharomyces*, *P.*: *Pichia*, *I.*: *Inosaccharomyces*, *C.*: *Candida*, *Sc.*: *Saccharomyces*, *Schiz.*: *Schizosaccharomyces*, *Lc.*: *Lactococcus*, *Lb.*: *Lactobacillus*, *Pd.*: *Pediococcus*, *A.*: *Aacetobacter*, *G.*: *Gluconobacter*, *B.*: *Bacillus*.

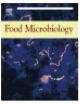
^a Detected sporadically.

^b Isolates did not grow upon purification. Identified directly by rep-PCR grouping and 16S rRNA gene sequencing (see text for details).

^c N.D.: None detected.

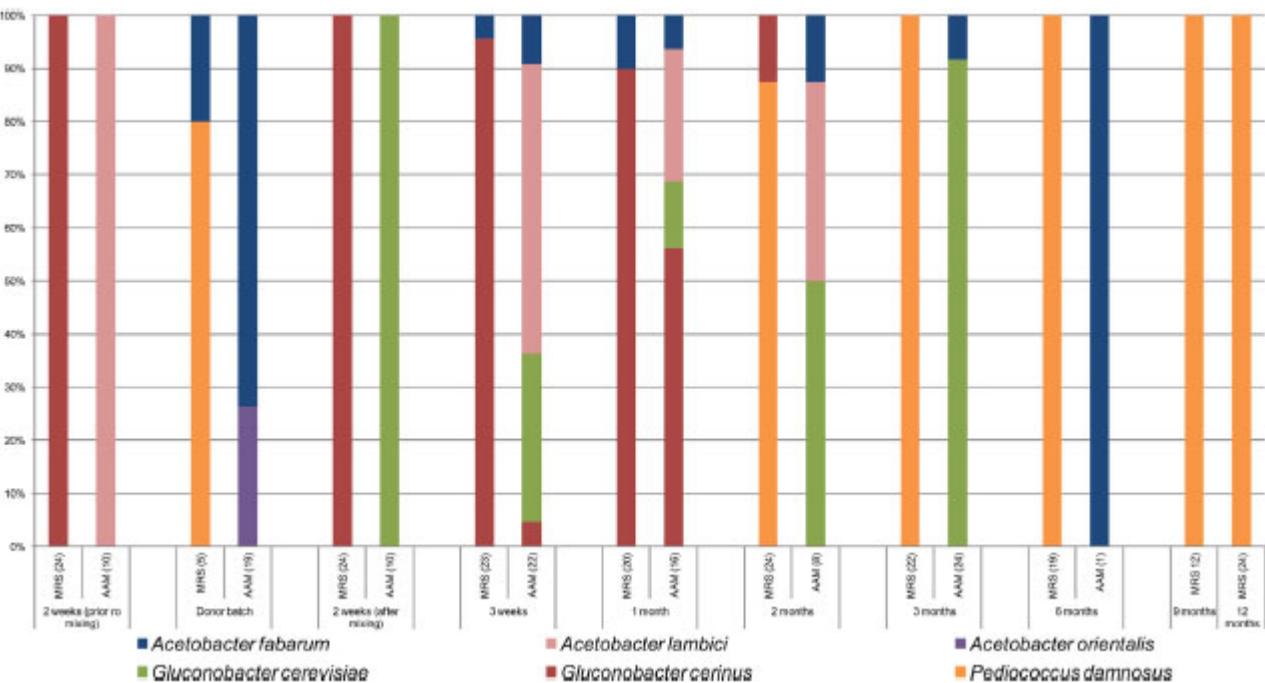
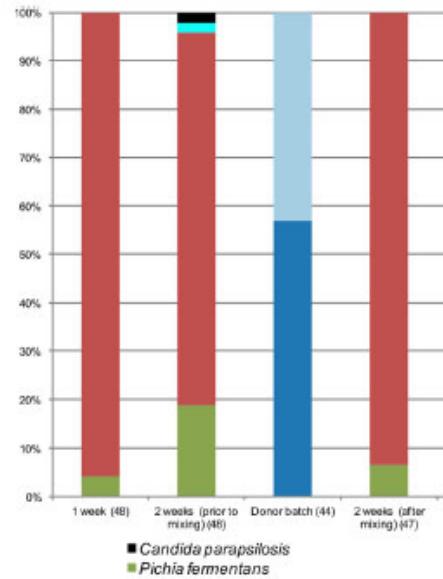
^d No data, plates partly overgrown by slimy non-AAB. CFU_{AAB} and AAB composition approximately as data for 60 h of fermentation.





Lambic

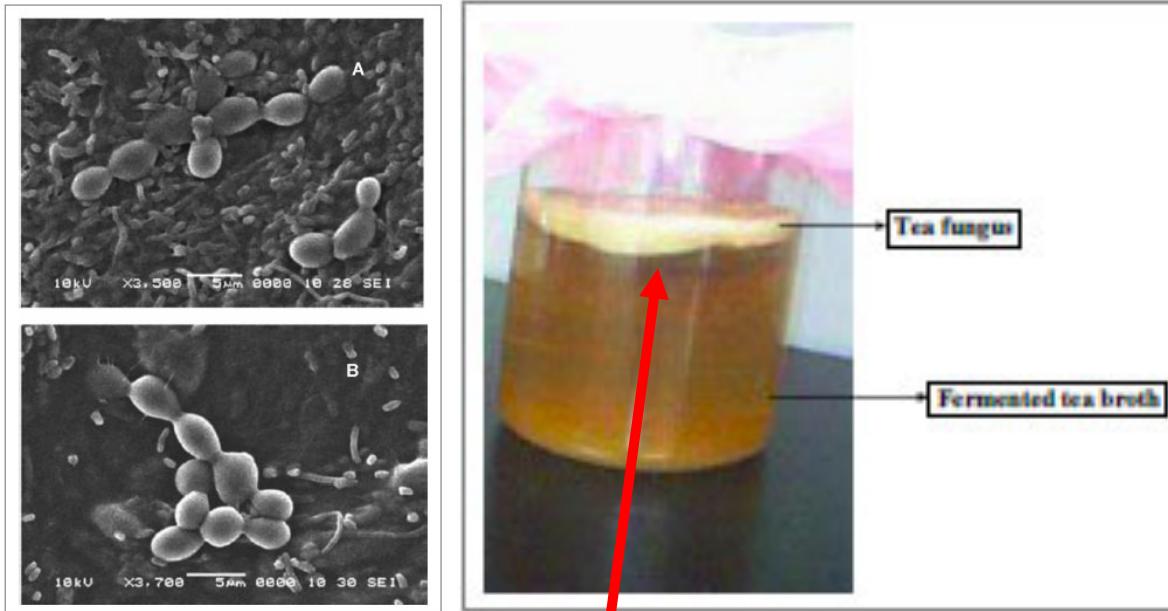
A combined yeast, LAB and AAB-fermentation



MFFB AAB
Dias 20

Kombucha

- Fermentation of sugared tea (sugar content 8-10%)
- The "tea fungus" is a mix of yeast (*Zygosaccharomyces* etc.) and AAB (*Komagatacibacter xylinum* and others) living in symbiosis in a "SCOPY"



SCOPY

A small assignment:
Explain what goes on in the SCOPY from a microbiological point of view?

- Who produces and metabolises what?
- What keeps the SCOPY together?
- Work in the same groups as before



Thanks

Questions?

dn@food.ku.dk



Gut microbiota, (fermented) food and human health

Dennis S. Nielsen, dn@food.ku.dk

KØBENHAVNS UNIVERSITET



Food – and their digestion



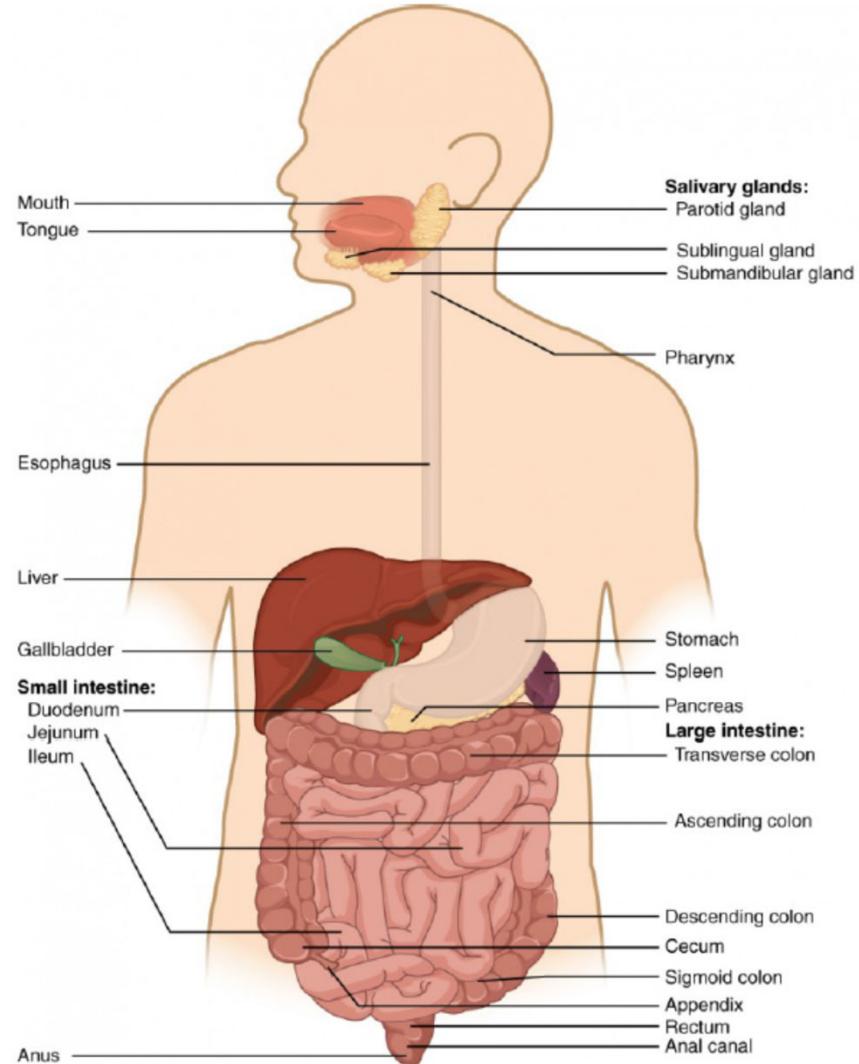
Different types of food have different composition

- Will this influence their digestion?
- If yes, how?

Our gastrointestinal system

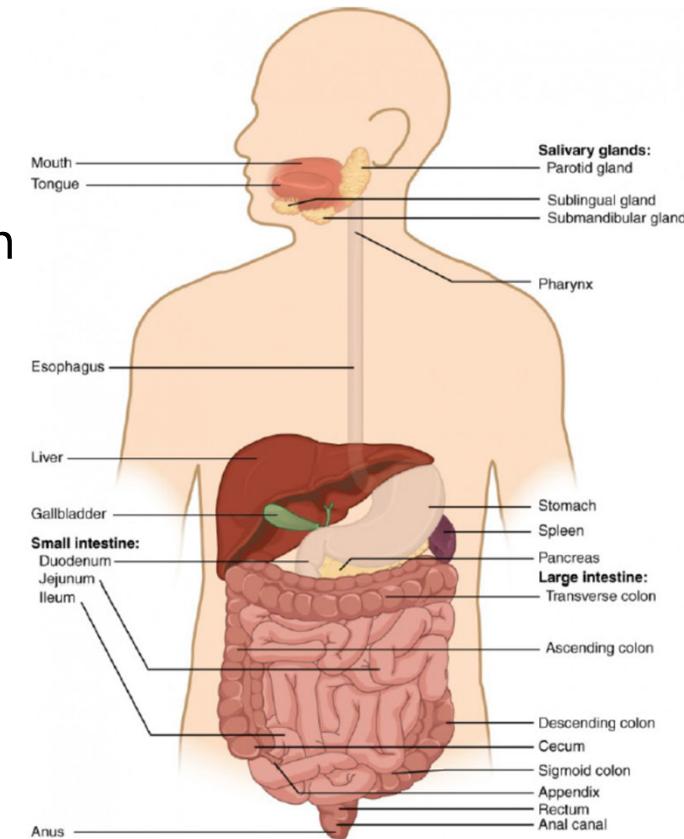
In short - the function of the digestive system is to:

- Break down the foods you eat,
- Release their nutrients
- Absorb those nutrients into the body

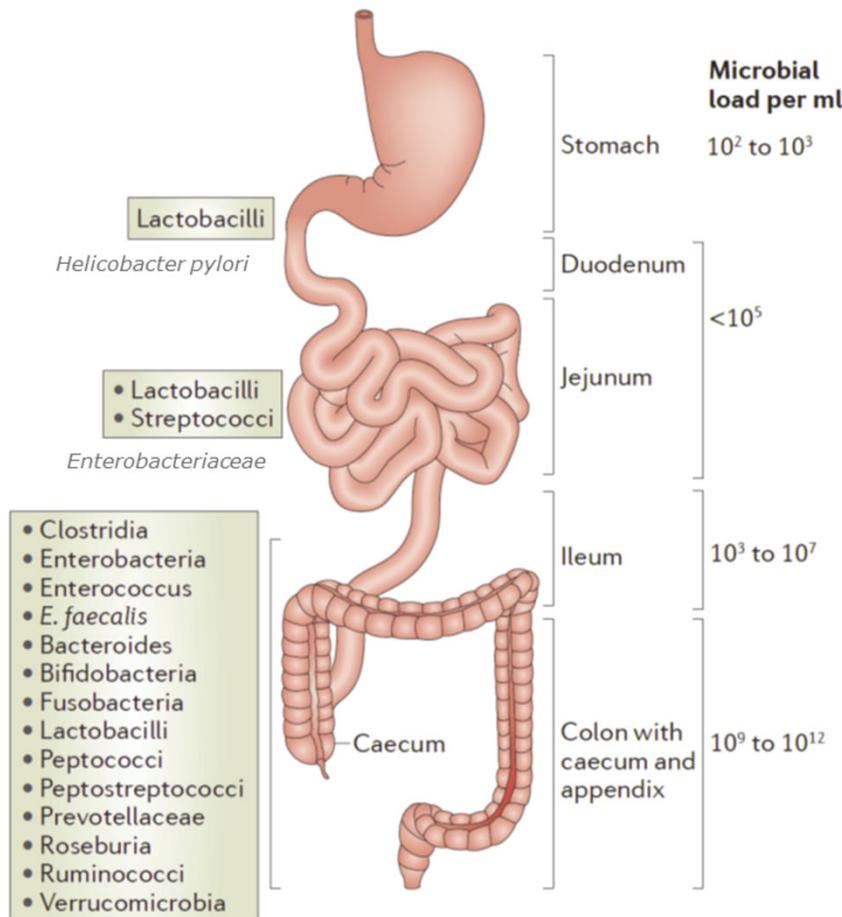


The organs of the digestive (/alimentary) tract

- The digestive tract: An open-ended tube with a total length of about 8–9 m, extending from mouth to anus
 - Pharynx
 - Esophagus
 - Stomach
 - Small intestine (duodenum, jejunum, ileum)
 - Large intestine (colon, rectum)
- Accessory organs: Teeth, tongue, salivary glands, liver, gall bladder, and pancreas



One GI tract, many microbiomes



Mouth:

- Enzymes (lysozyme)
- Oxygen

Stomach

- Some oxygen
- Gastric enzymes
- Low pH

Small intestine

- Bile, bile, bile
- Gastric enzymes
- (Virtually) no oxygen

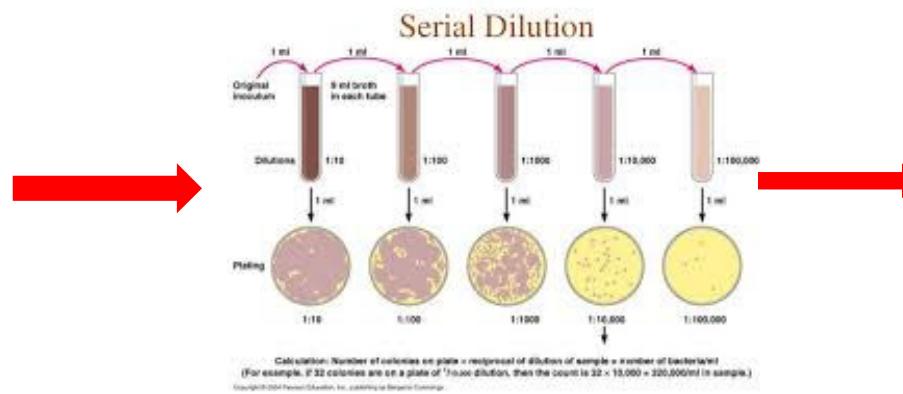
Colon

- Anaerobic
- All the easy stuff have been absorbed
 - Nondigestable carbohydrates
 - Some protein (especially of plant origin)

Mowat & Agace, Nature Rev. Immunol, 2014

GM characterization by cultivation based techniques

- Cultivation based techniques



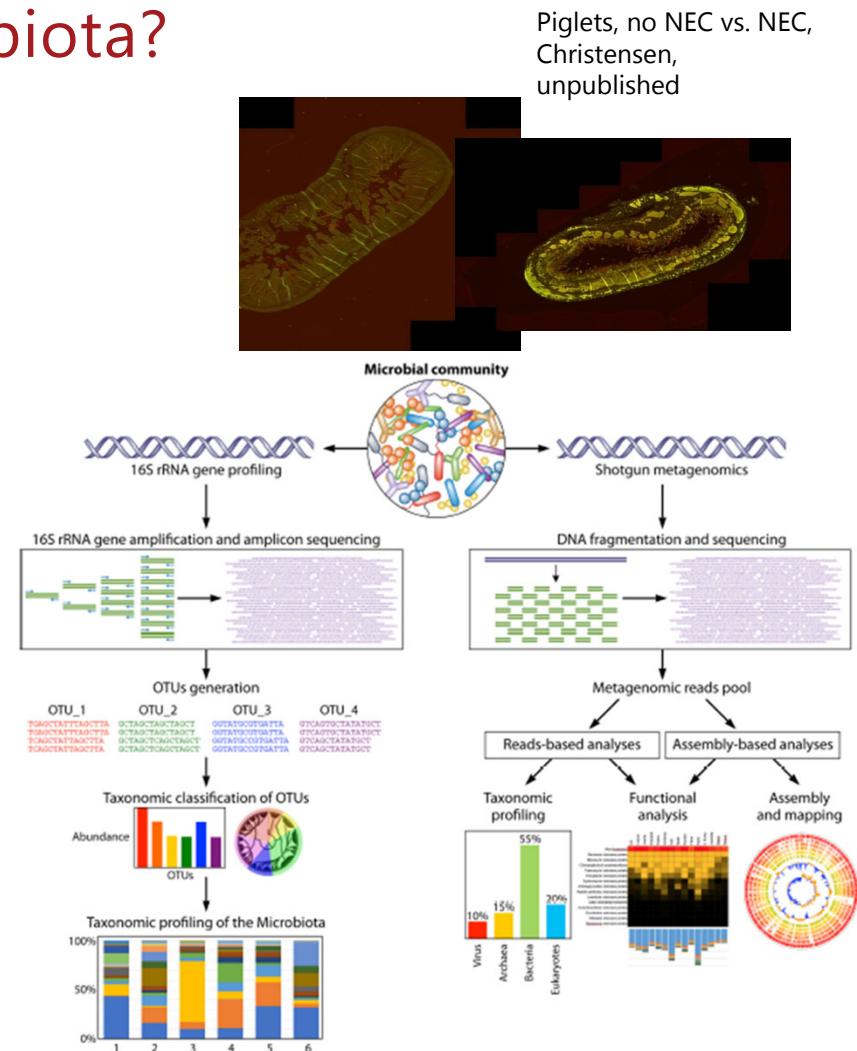
Identification.
Today often
based on 16S
rRNA gene (or
other genes)
sequencing
and/or MALDI-
TOF

- But each individual harbour 200-500 different species in their GM...
 - Many different substrates needed for their cultivation
- Many species difficult/impossible to cultivate (obligate anaerobes)



How to characterise the gut microbiota?

- **Culture-independent techniques**
- FISH and "FISH-like"
- qPCR (targeted, absolute quantification)
- High throughput sequencing (HTS)
 - Tag-encoded amplicon-seq. (e.g. 16S rRNA and ITS-genes; 20-30 Euros/sample) => "who is there"
 - Metagenome (100-200 Euros/sample) ("who" and "what can they do")
 - RNAseq (which genes are expressed)
 - Meta-virome (all viruses incl. bacteriophages)



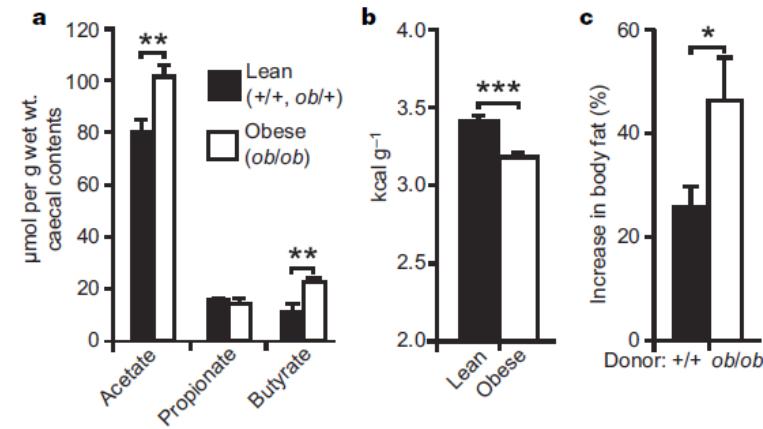
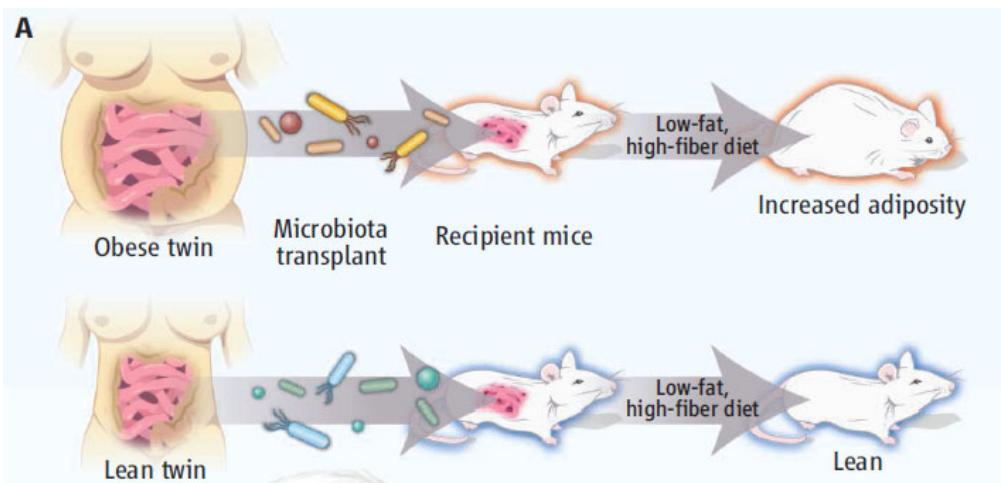
Gut microbiota and obesity

- The fuzz about GM started with some obese mice 18 years ago

Obesity alters gut microbial ecology

PNAS | August 2, 2005 | vol. 102
Ruth E. Ley[†], Fredrik Bäckhed[†], Peter Turnbaugh[†], Catherine A. Lozupone[‡], Robin D. Knight[§], and Jeffrey I. Gordon^{†¶}

- Then it was shown that the obese GM has an increased capacity for energy harvest
- And that the obese phenotype is transferable with the GM



An obesity-associated gut microbiome with increased capacity for energy harvest

Peter J. Turnbaugh¹, Ruth E. Ley¹, Michael A. Mahowald¹, Vincent Magrini², Elaine R. Mardis^{1,2} & Jeffrey I. Gordon¹

Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice

Vanessa K. Ridaura,¹ Jeremiah J. Faith,¹ Federico E. Rey,¹ Jiye Cheng,¹ Alexis E. Duncan,^{2,3} Andrew L. Kau,² Nicholas W. Griffin,¹ Vincent Lombard,⁴ Bernard Henrissat,^{4,5} James R. Bain,^{6,7,8} Michael J. Muehlbauer,⁶ Olga Ilkayeva,⁹ Clay F. Semenkovich,⁹ Katsuhiko Funai,⁹ David K. Hayashi,¹⁰ Barbara J. Iglesias,¹¹ Margaret C. Martini,¹¹ Luke K. Ursell,¹² Jose C. Clemente,¹² William Van Treuren,¹² William A. Walters,¹³ Rob Knight,^{12,14,15} Christopher B. Newgard,^{6,7,8} Andrew C. Heath,² Jeffrey I. Gordon^{1*}

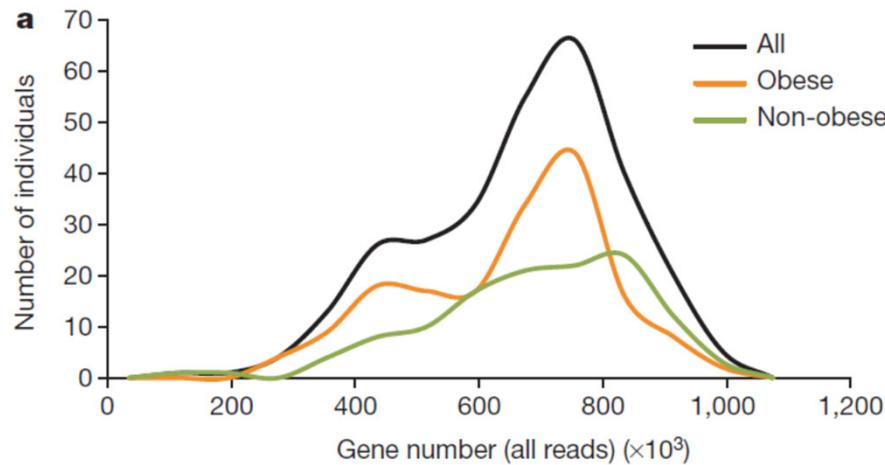
What do know today?

- Obesity, metabolic syndrome, type 2 diabetes
- Asthma, eczema, type 1 diabetes (autoimmune diseases)
- Inflammatory bowel disease
- Colon cancer
- Cardiovascular disease
- Autism
- Liver disease
- Behaviour/depression
- Etc. etc.

The healthy gut microbiome

Overall the healthy microbiome is characterised by

- High taxa diversity
 - Sometimes...
- High microbial gene richness
- Stable microbiome functional cores



A core gut microbiome in obese and lean twins

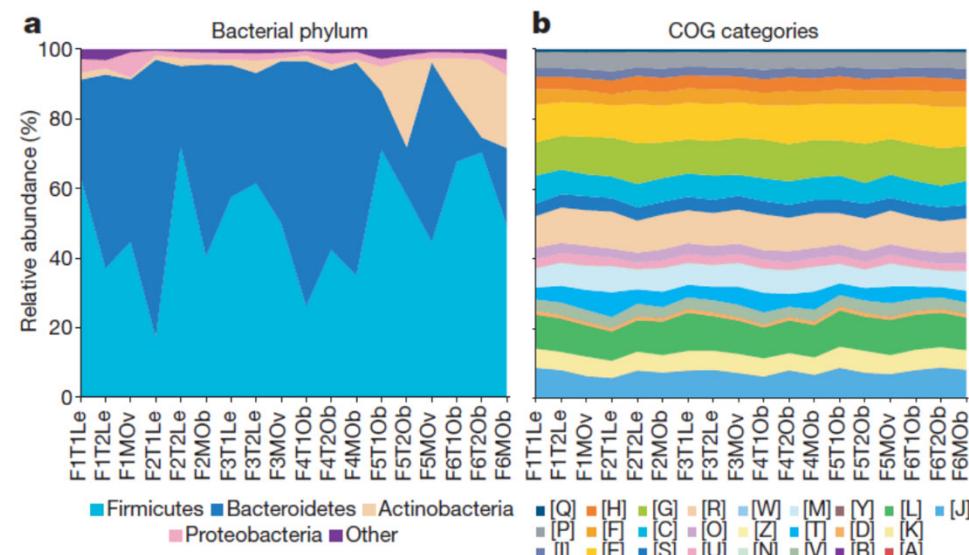
Peter J. Turnbaugh¹, Micah Hamady³, Tanya Yatsunenko¹, Brandi L. Cantarel⁵, Alexis Duncan², Ruth E. Ley¹, Mitchell L. Sogin⁶, William J. Jones⁷, Bruce A. Roe⁸, Jason P. Affourtit⁹, Michael Egholm⁹, Bernard Henrissat⁵, Andrew C. Heath², Rob Knight⁴ & Jeffrey I. Gordon¹

NATURE | Vol 457 | 22 January 2009

Richness of human gut microbiome correlates with metabolic markers

Emmanuelle Le Chatelier^{1,8}, Trine Nielsen^{2,8}, Junjie Qin^{1,8}, Edi Prifti¹, Falk Hildebrand^{4,5}, Gwen Falony^{4,5}, Mathieu Almeida¹, Manimozhyan Arumugam^{3,1,2}, Jean-Michel Batté³, Sean Kennedy³, Pierre Leonard³, Junhua Li^{1,2}, Kristoffer Burgdorf², Niels Grarup², Torben Jørgensen^{4,8,9,10}, Ivan Brändsköld^{1,12}, Henrik Bjørn Nielsen¹², Agnieszka S. Juncker¹², Marcelo Bertalan¹², Florence Levenez¹, Nicolas Pons¹, Simon Rasmussen^{1,3}, Shinichi Sunagawa¹, Jülien Tap^{1,8}, Sebastian Timms¹, Erwin G. Zoetendal¹², Søren Brunak¹², Karine Clement^{1,2}, Jérôme Danchin¹², Mikkel Zondervan^{1,2}, Karsten Kristoffersen¹, Pierre Kehat¹¹, Thomas Sicheritz-Ponten¹, Willems B. de Vos^{14,20}, Jean-Daniel Zuccaro^{15,16,21}, Jeroen Raes^{4,5,1}, Peer Bork¹, Jun Wang^{19,23,24,25}, S. Dusko Ehrlich¹ & Oluf Pedersen^{26,27,28}, MetaHIT consortium, Peer Bork¹, Jun Wang^{19,23,24,25}, S. Dusko Ehrlich¹ & Oluf Pedersen^{26,27,28}, MetaHIT

29 AUGUST 2013 | VOL 500 | NATURE | 541



COG = Clusters of Orthologous Genes

How to “make” an healthy/unhealthy GM

Diet is the major driver of GM composition/function

Abbreviations:

GPCR = G-protein coupled receptor

PPAR γ = peroxisome

proliferator-activated receptor- γ

PYY = peptide YY

GLP1 = Glucagon-like peptide 1

PAMP = Pathogen-associated microbial pattern

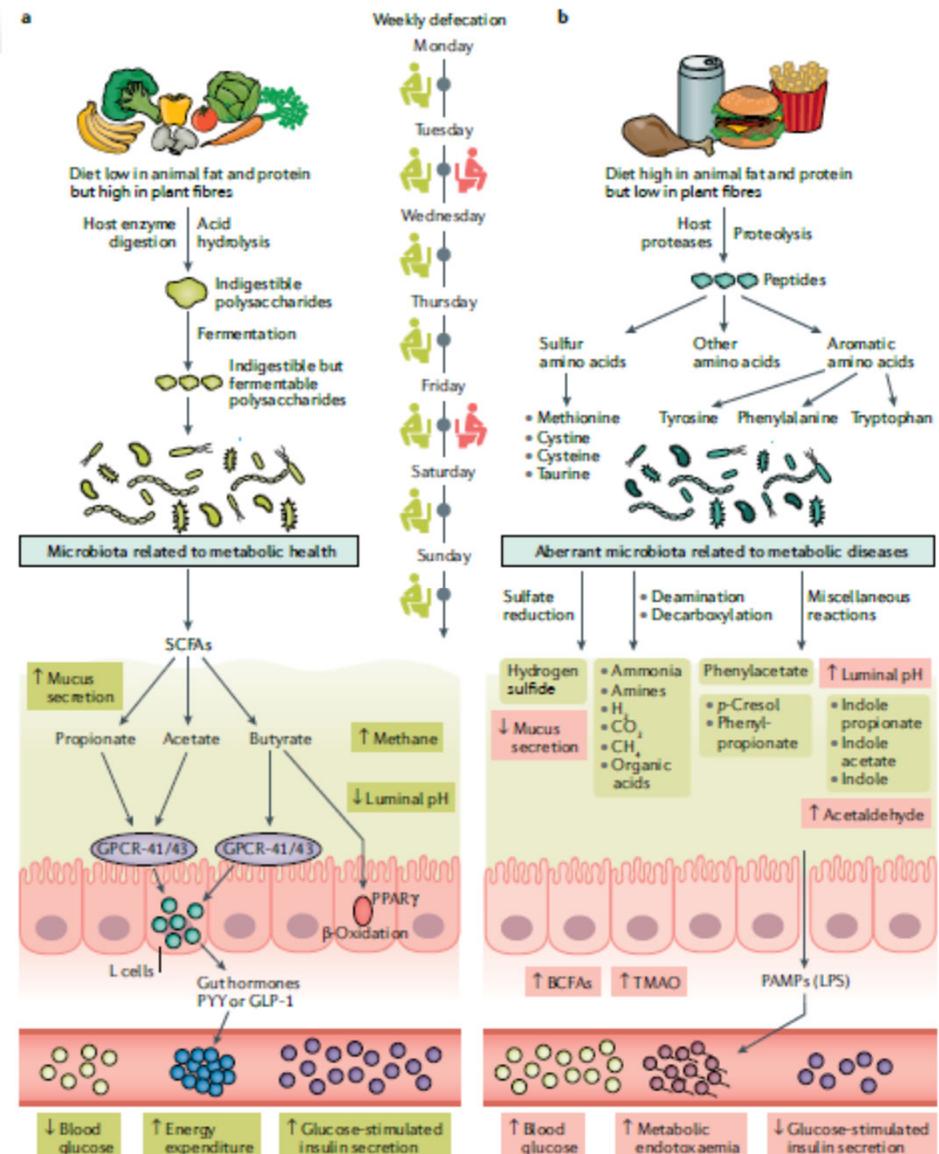
TMAO = trimethylamine N-oxide

Gut microbiota in human metabolic health and disease

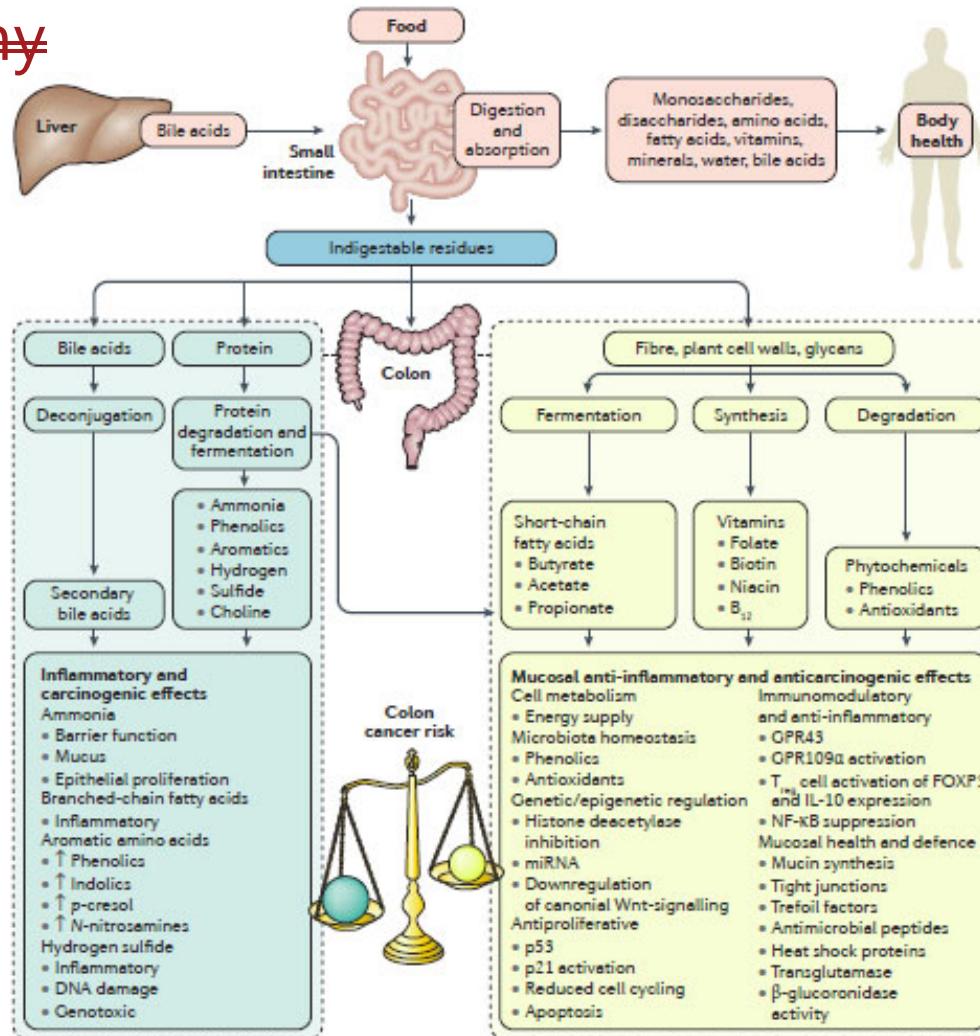
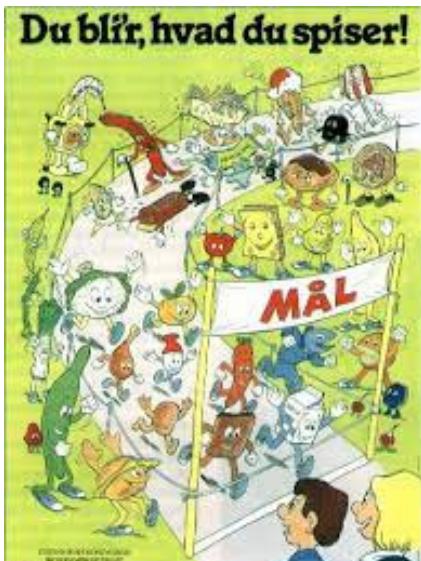
Yong Fan and Oluf Pedersen

NATURE REVIEWS | MICROBIOLOGY

VOLUME 19 | JANUARY 2021



It's the ~~economy~~ diet, stupid

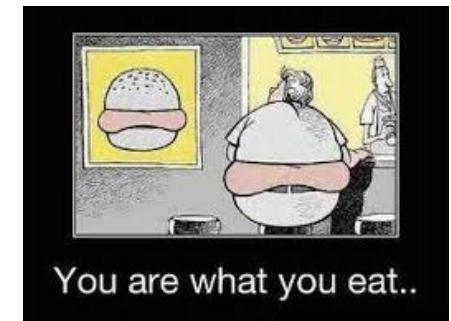


Diet, microorganisms and their metabolites, and colon cancer

Stephen J. D. O'Keefe

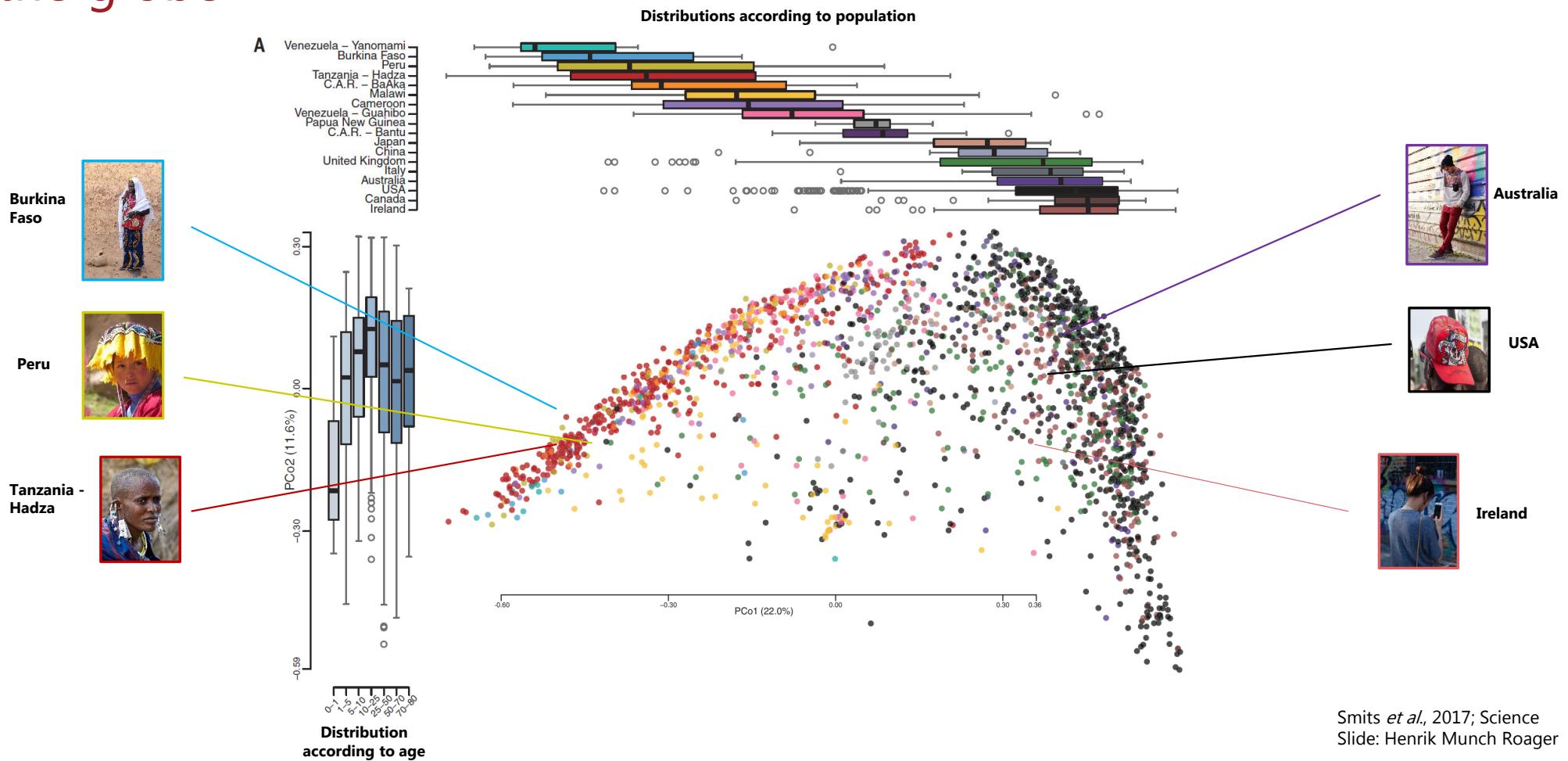
NATURE REVIEWS | GASTROENTEROLOGY & HEPATOLOGY

VOLUME 13 | DECEMBER 2016 | 691



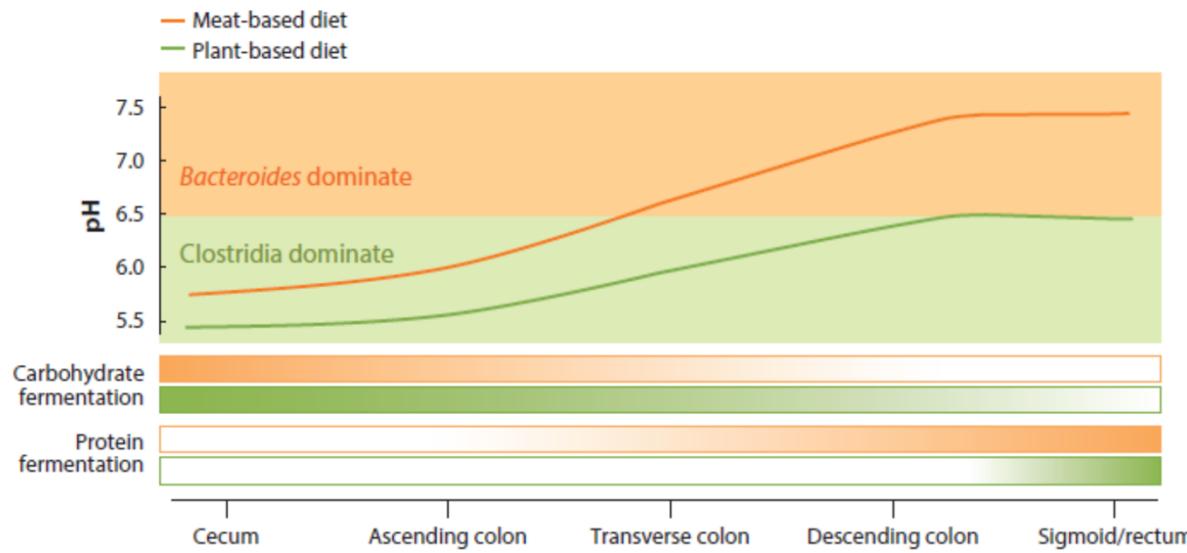
Bray-Curtis dissimilarity PCoA
based on 2064 microbial
community compositions
described at the family
taxonomic level across
populations

The adult gut microbiome composition across the globe



Degradation of dietary fibre

- **Dietary fibre – non-digestible carbohydrates**
 - However, some fibres are not used by gut microbes (cellulose)
 - Some complex fermentable carbohydrates are not defined as fibres (e.g. resistant starch)
- **Microbiota-accessible carbohydrates (MAC)**
 - Carbohydrates metabolically available to gut microbes

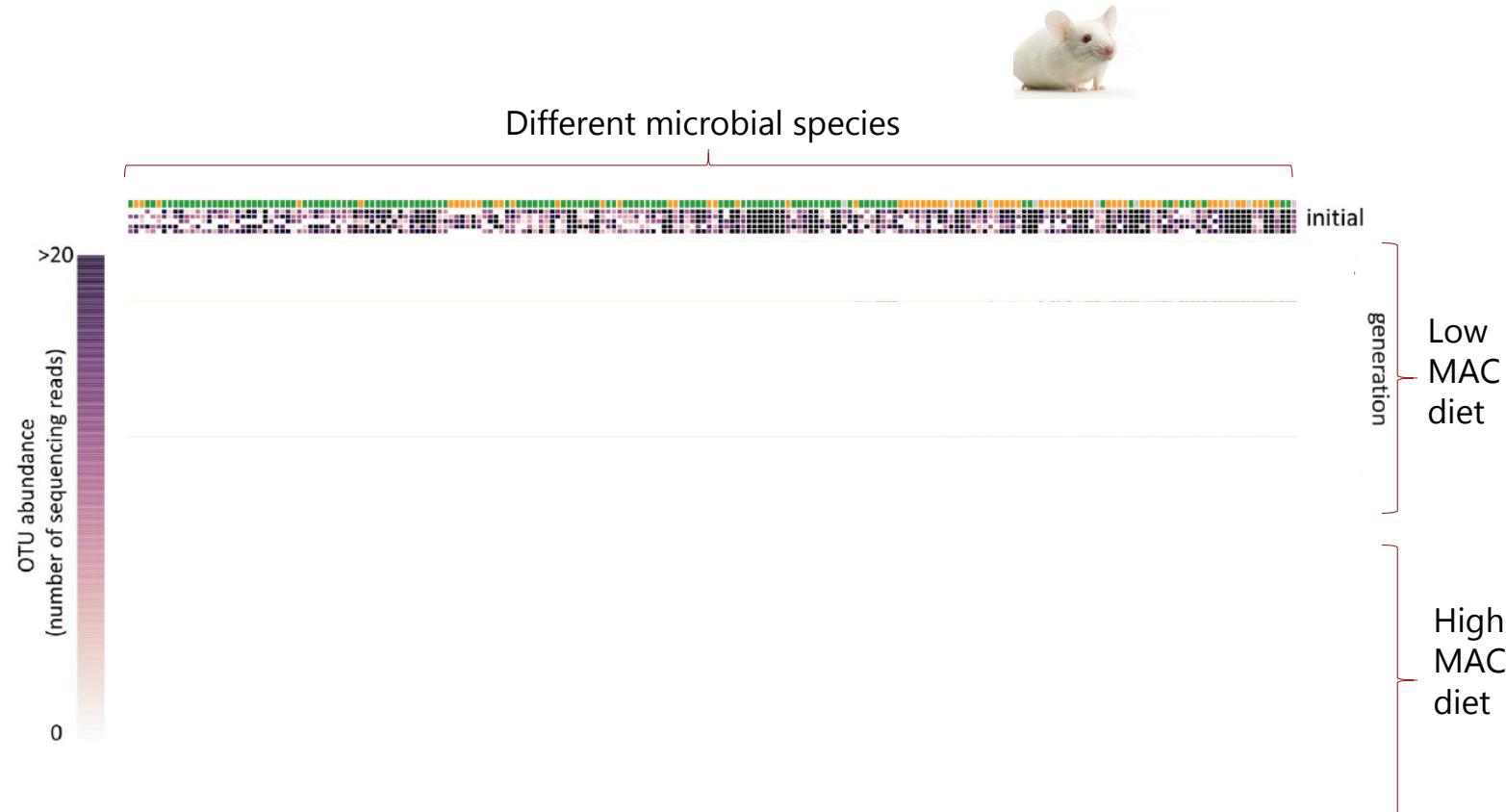


Annual Review of Food Science and Technology
Diet, Microbiota, and
Metabolic Health: Trade-Off
Between Saccharolytic and
Proteolytic Fermentation

Katri Korpela^{1,2}

Partly adapted from Henrik
Munch Roager

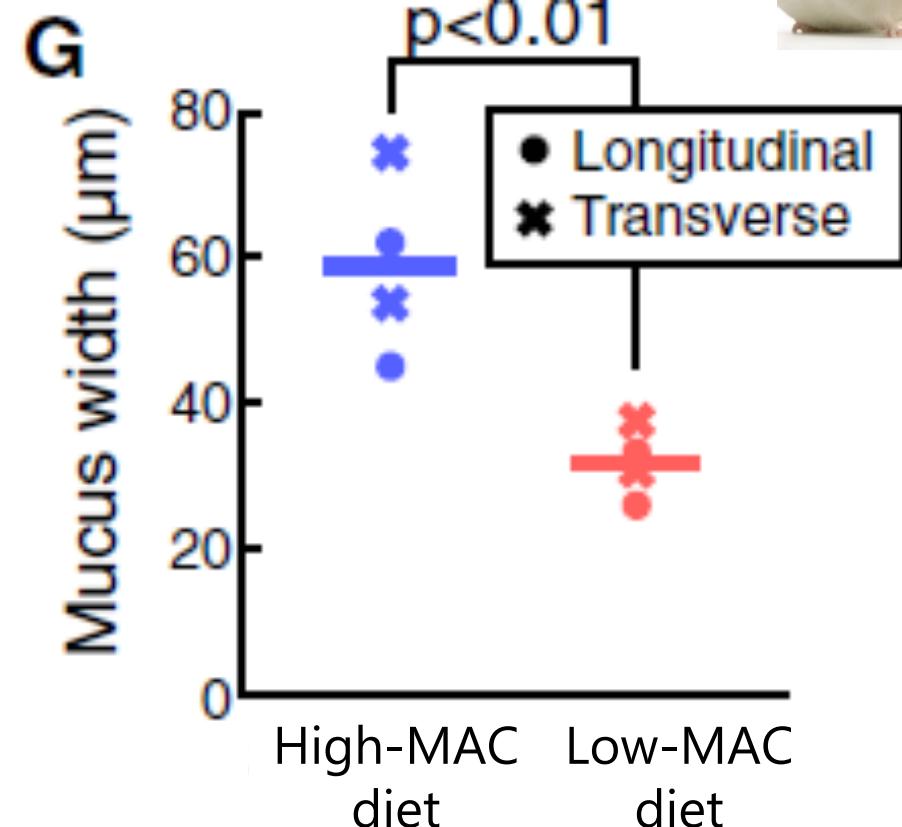
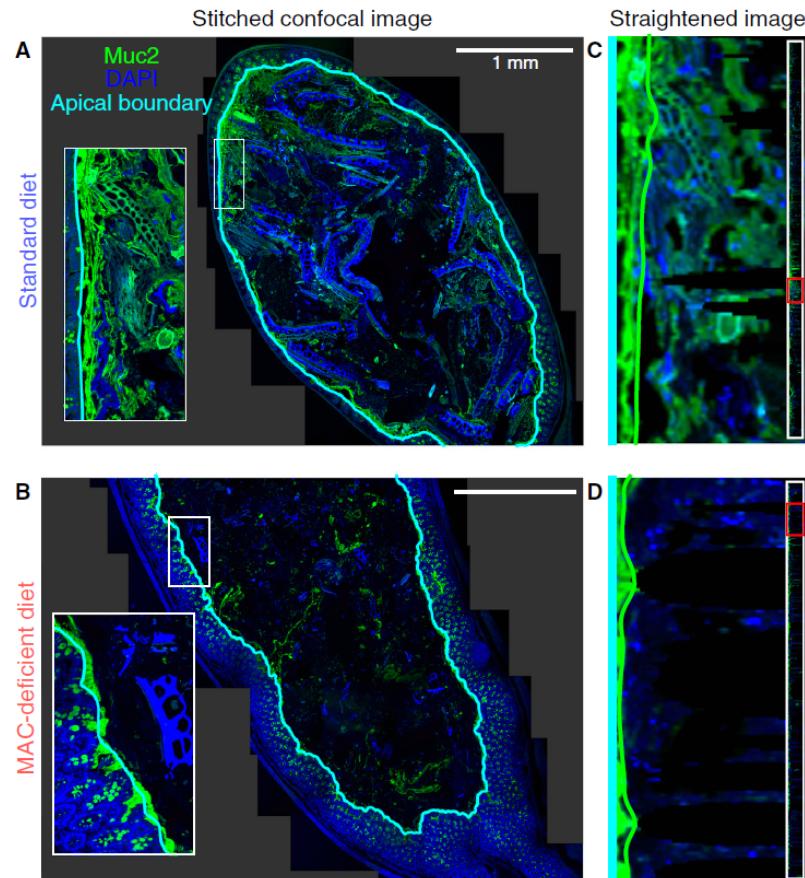
The importance of microbiota-accessible carbohydrates (MAC)



Sonnenburg *et al.*, 2016; Nature

Courtesy of Henrik Munch Roager

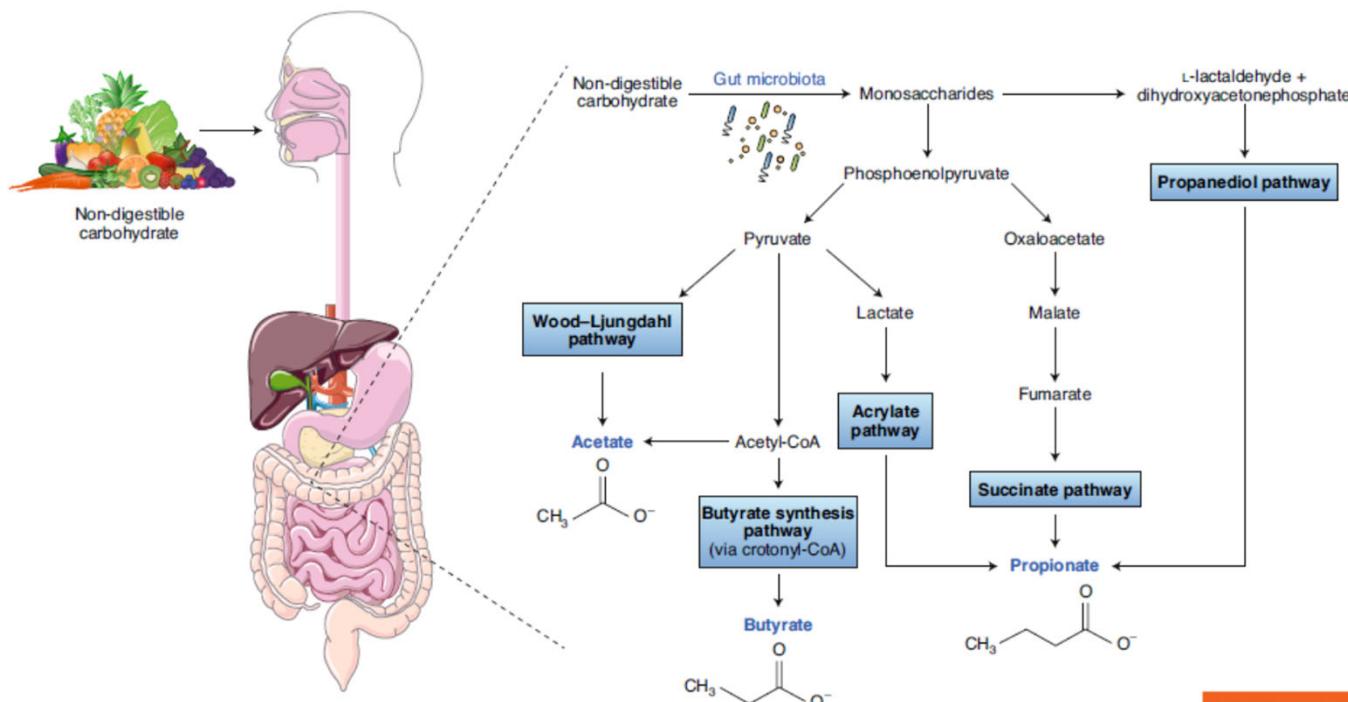
Consequences of reduced intake of MAC



Courtesy of Henrik
Munch Roager

Earle *et al.*, 2015; Cell Host & Microbe

Degradation of complex carbohydrates – and formation of short chain fatty acids in the colon



- Non-digestable carbohydrates (fibre, resistant starch) **main** source of SCFA
- Protein fermentation will also lead to SCFA
 - And some less nice compounds
- Mucin also a source of SCFA

REVIEW ARTICLE
<https://doi.org/10.1038/naturemetabolism.2020-0186-7>

nature
metabolism

Check for updates

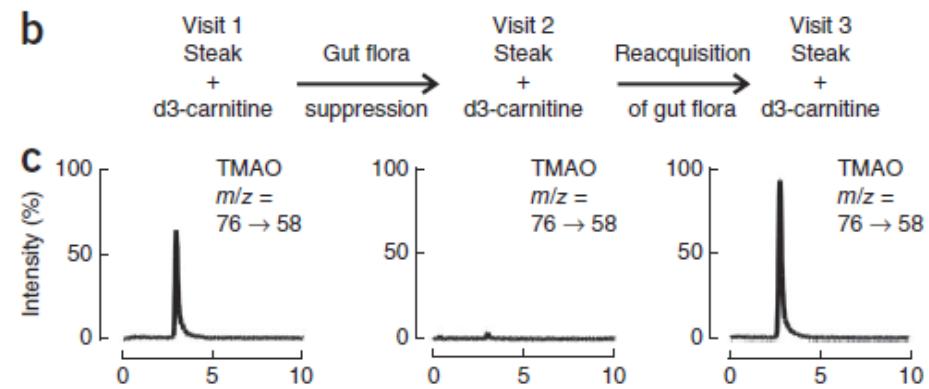
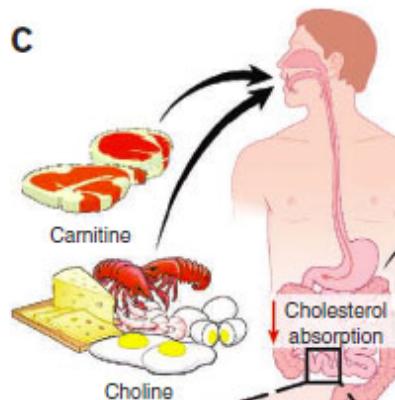
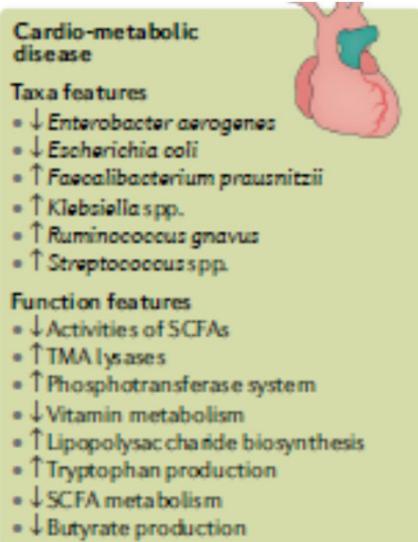
Short-chain fatty acids as potential regulators of skeletal muscle metabolism and function

James Frampton^{1,2}, Kevin G. Murphy², Gary Frost³ and Edward S. Chambers^{1,2}

Cardiometabolic disease and the GM

Du bliver, hvad du spiser (you become what you eat)

- Cardio-metabolic disease, CMD (or CVD), is linked to GM dysbiosis and certain microbial metabolites a risk factor
- High plasma levels of TMAO (trimethylamine-N-oxide) = increased risk of CVD
- Driven jointly by diet and GM



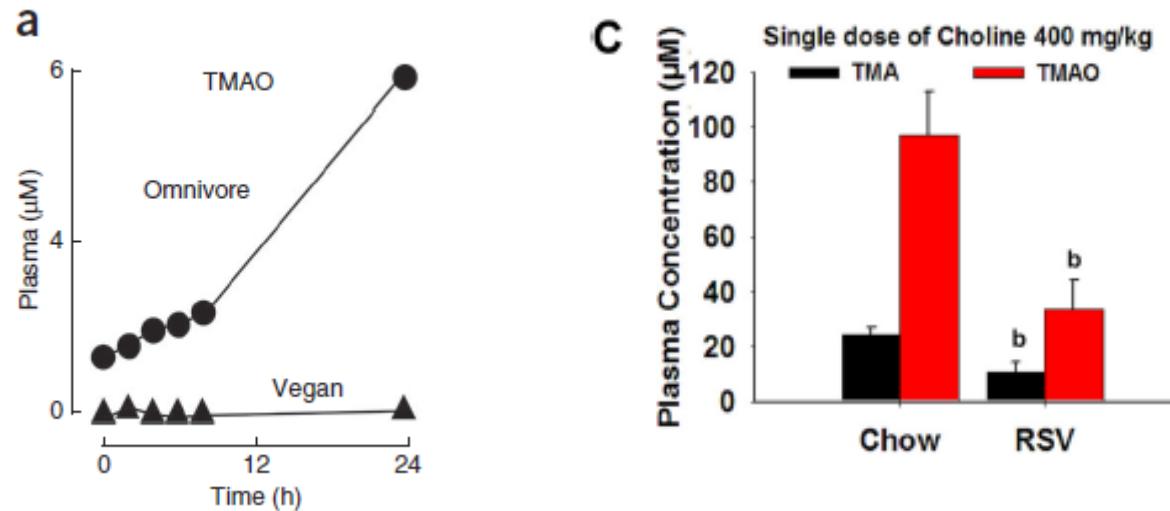
Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis

Robert A Koeth^{1,2}, Zeneng Wang^{1,2}, Bruce S Levison^{1,2}, Jennifer A Buffa^{1,2}, Elin Org³, Brendan T Sheehy¹, Earl B Britt^{1,2}, Xiaoming Fu^{1,2}, Yuping Wu⁴, Lin Li^{1,2}, Jonathan D Smith^{1,2,5}, Joseph A DiDonato^{1,2}, Jun Chen⁶, Hongzhe Li⁶, Gary D Wu⁷, James D Lewis^{8,9}, Manya Warrier⁹, J Mark Brown⁹, Ronald M Krauss¹⁰, W H Wilson Tang^{1,2,5}, Frederic D Bushman⁵, Aldons J Lusis³ & Stanley L Hazen^{1,2,5}

Cardiometabolic disease and the GM

Du bliver, hvad du spiser (you become what you eat)

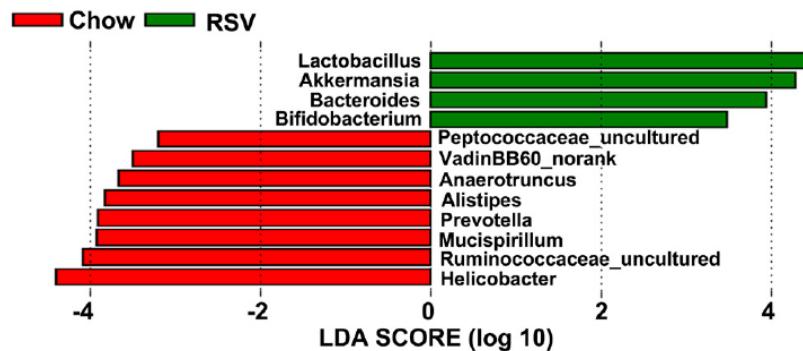
- High plasma levels of TMAO (trimethylamine-N-oxide) = increased risk of CVD/CMD
- Driven jointly by diet and GM
- Vegetarians and vegans do not form TMAO, as their GM has not been exposed to these substrates for a long time
- Some dietary components are able to limit TMAO-formation, e.g. resveratrol



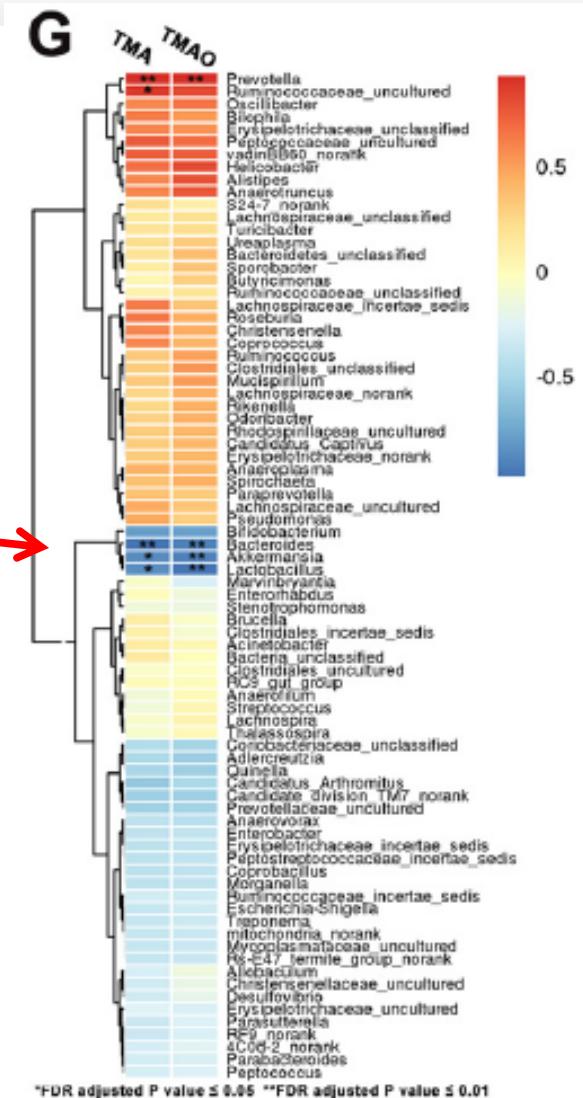
Cardiometabolic disease and the GM

Du bliver, hvad du spiser (you become what you eat)

- The protective effect of resveratrol against TMAO-formation is associated with GM changes

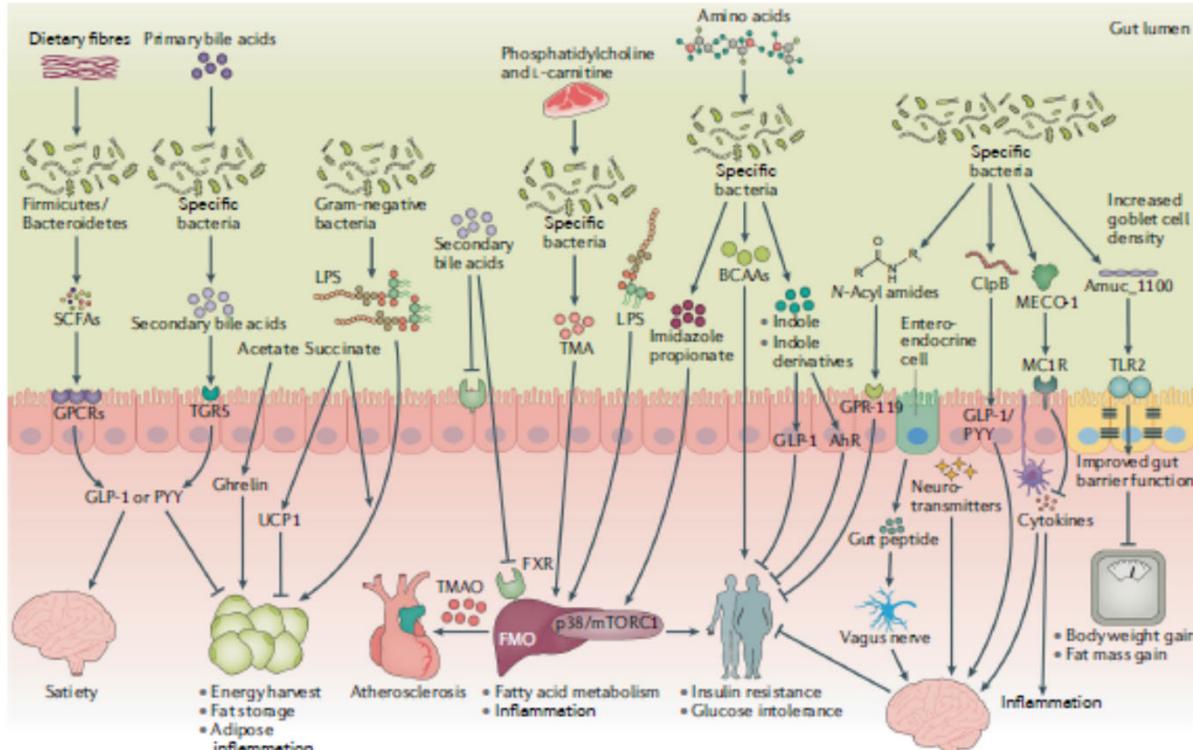


- Which in turn lower TMAO-formation



*FDR adjusted P value ≤ 0.05 **FDR adjusted P value ≤ 0.01

Microbial messengers regulate host metabolism



- TGR5 = Takeda G protein-coupled receptor, specific for bile acids, promote carbohydrate metabolism
- UCP1 = uncoupling protein 1, increases thermogenesis in adipose tissue (expression increased by succinate)
- LPS = Lipopolysaccharide, trigger inflammation and insulin resistance. Succinate increase inflammatory response
- FXR = Farnesoid X receptor, impair carbohydrate metabolism
- FMO = Flavin-containing monooxygenase, Trimethylamine (TMA) => trimethylamine N-oxide (TMAO)
- Imidazole propionate, worsen glucose insensitivity
- Indole + derivatives, bind to aryl hydrocarbon receptor (AhR). Indole improve barrier function + signal release of GLP-1. But will also be converted to indoxyl sulphate in the liver (CMD)
- ClpB = a bacterial chaperone and a antigen-mimetic of α -melanocyte-stimulating hormone implicated in body weight regulation
- MECO-1 = Melanocortin-like peptide, inhibits cytokine release

Microbial metabolites function as "messengers" regulating host metabolism

- Many are formed by the colonic microbiota from undigested food components
- But many will also be present in fermented foods in high amounts
 - Lactate, acetate, propionate
 - Succinate
 - Formation of aromatic metabolites in polyphenol rich foods
 - Vitamins
 - γ -aminobutyric acid
 - Etc. etc.

Gut microbiota in human metabolic health and disease

Yong Fan and Oluf Pedersen

NATURE REVIEWS | MICROBIOLOGY
VOLUME 19 | JANUARY 2021

Fermented foods and the gut

- An unbalanced/dysbiotic GM often lack certain functions
=> E.g. reduced capability of butyric acid production
- Fermented foods contain many (often live) microbes
=> Will intake of fermented foods positively influence your GM and gut health?



Kombucha (fermented tea), 1-10 million microbes pr. gram



Yoghurt (fermented milk), 10 million microbes pr. gram



Sauerkraut (fermented white cabbage), 1-10 million microbes pr. gram

Kimchi and the GM

- Kimchi is the Korean version of sauerkraut
 - White cabbage, salt, spices (chili etc.)
 - Contain 1-50 million microbes/gram (mainly lactic acid bacteria)
- Can intake of kimchi improve disease symptoms of diseases associated with GM dysbiosis?

Table 4
Changes in clinical and anthropometric parameters

	Baseline ^a (n = 22)	Fresh kimchi (n = 22)		Fermented kimchi (n = 22)	
		Initial ^b	Final ^c	Initial ^d	Final ^e
Body weight (kg)	73.6 ± 9.9	72.9 ± 9.6	71.7 ± 9.4 *	73 ± 10.1	71.5 ± 9.7 *
BMI (kg/m ²)	27.7 ± 2.0	27.4 ± 2.2	27.0 ± 2.2 *	27.5 ± 2.2	26.9 ± 2.2 *
WHR	0.87 ± 0.53	0.86 ± 0.05	0.85 ± 0.06	0.86 ± 0.06	0.84 ± 0.06 *
Body fat (%)	32.7 ± 3.8	31.9 ± 4.0	31.6 ± 4.0 *	32.1 ± 4.3	31.4 ± 4.4 ***
Systolic BP (mm Hg)	128.7 ± 11.7	125.8 ± 10.7	122.1 ± 7.9	126.1 ± 12.1	121.3 ± 6.9 **
Diastolic BP (mm Hg)	78.6 ± 10.4	76.1 ± 9.9	74.7 ± 8.5	76.9 ± 9.7	72.7 ± 7.4 **
Total cholesterol (mg/dL)	177 ± 25.7	176 ± 29.5	172 ± 31.6	171 ± 25.7	161 ± 29.9 * ***

- Conclusion: It is good to eat kimchi. Eating fermented kimchi is even better

Fermented kimchi reduces body weight and improves metabolic parameters in overweight and obese patients

Eun Kyung Kim^{a,1}, So-Yeon An^{a,1}, Min-Seok Lee^{a,1}, Tae Ho Kim^b, Hye-Kyung Lee^c, Won Sun Hwang^c, Sun Jung Choe^c, Tae-Young Kim^d, Seung Jin Han^a, Hae Jin Kim^a, Dae Jung Kim^a, Kwan-Woo Lee^{a,*}

Sauerkraut and IBS

- Irritable bowel syndrome (IBS) is associated with gastrointestinal discomfort and GM dysbiosis
- Sauerkraut contains many potentially beneficial microbes – mainly lactic acid bacteria
- Will sauerkraut intake alleviate IBS symptoms?
- Intervention study carried out by 3 students
 - 6 week ingesting either pasteurised or non-pasteurised sauerkraut, 75 g/day (60 IBS patienter)



Eirik



Kathrine



Elsa



Salt,
fermentation

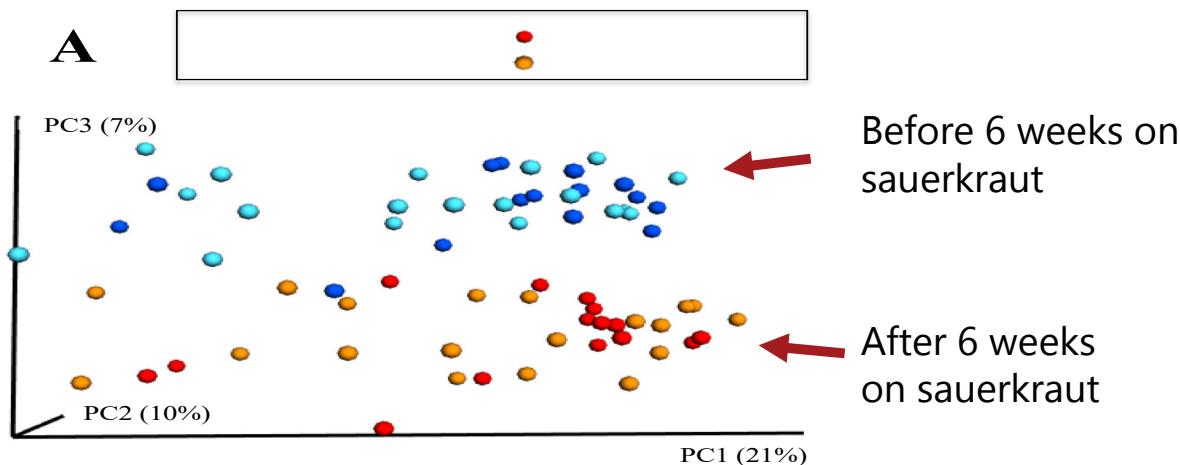


Pasteurised

Not
pasteurised

Sauerkraut and IBS

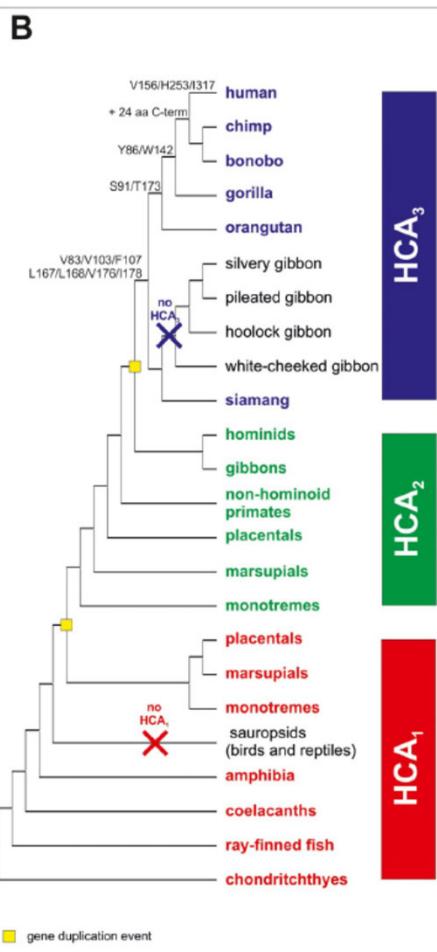
- It works. IBS-score reduced by 20% after intervention (in both groups)
- Strong effect of sauerkraut on the GM



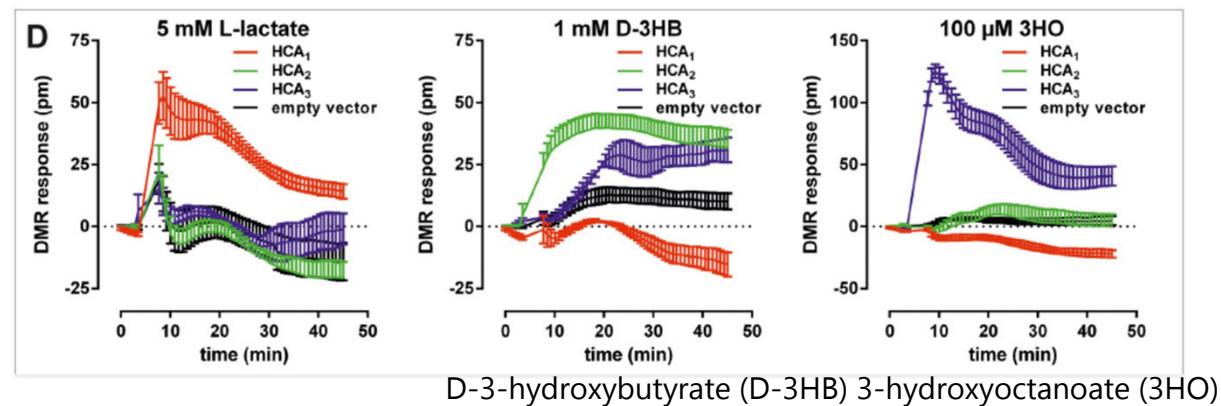
- But no difference between fresh and pasteurised sauerkraut
 - *In other words: It is not the living microbes that does the trick. Instead it is the good stuff from the cabbage + all the metabolites being produced during fermentation that positively influence IBS symptoms*

Nielsen et al. (2018), Food & Function

How do metabolites signal to us?



- HCA = hydrocarboxylic acid receptor
 - A GPCR (G-protein coupled receptor)
 - HCA3 only in great apes and humans



- Specific compounds act as agonists

PLOS GENETICS

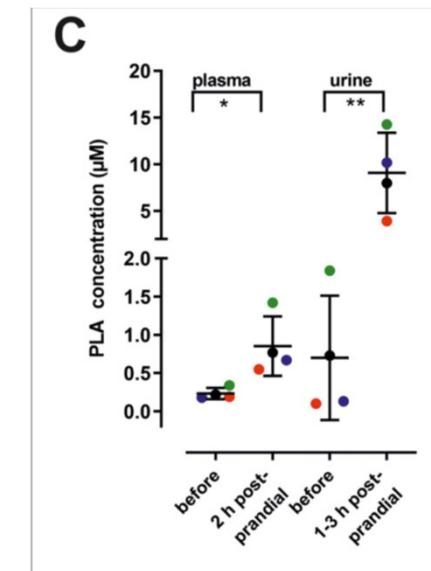
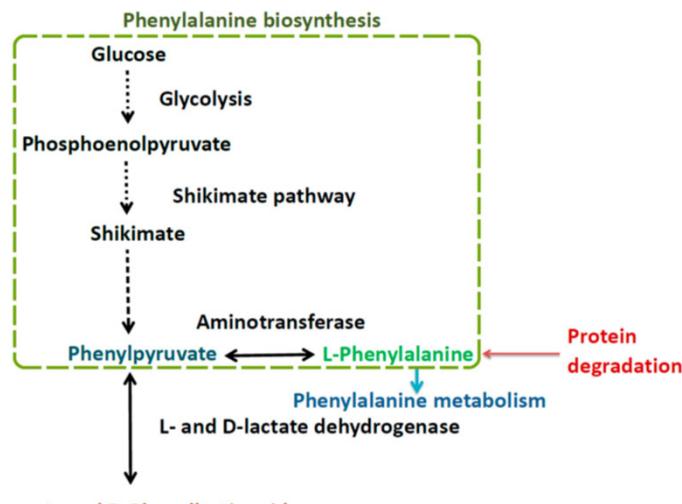
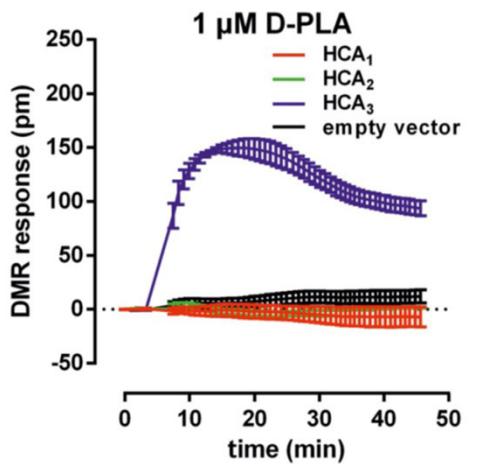
RESEARCH ARTICLE

Metabolites of lactic acid bacteria present in fermented foods are highly potent agonists of human hydroxycarboxylic acid receptor 3

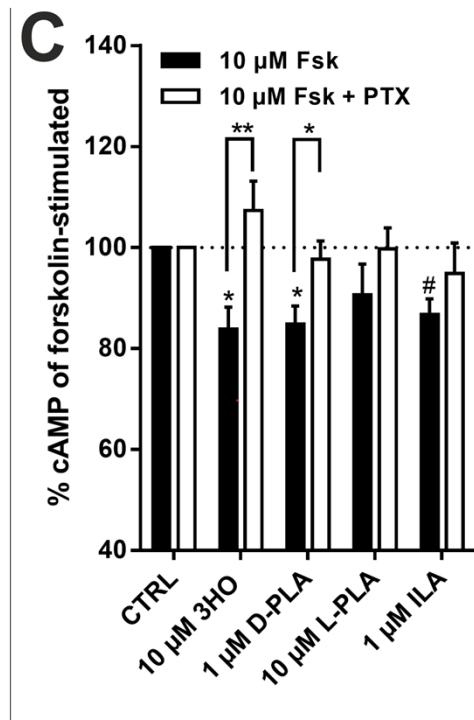
Anna Peters¹, Petra Krumbholz¹, Elisabeth Jäger², Anna Heintz-Buschart^{3,4}, Mehmet Volkan Çakir³, Sven Rothemund³, Alexander Gaudi³, Uta Ceglarek³, Torsten Schönberg¹, Claudia Stäubert^{3,4*}

How does fermented influence the immune system

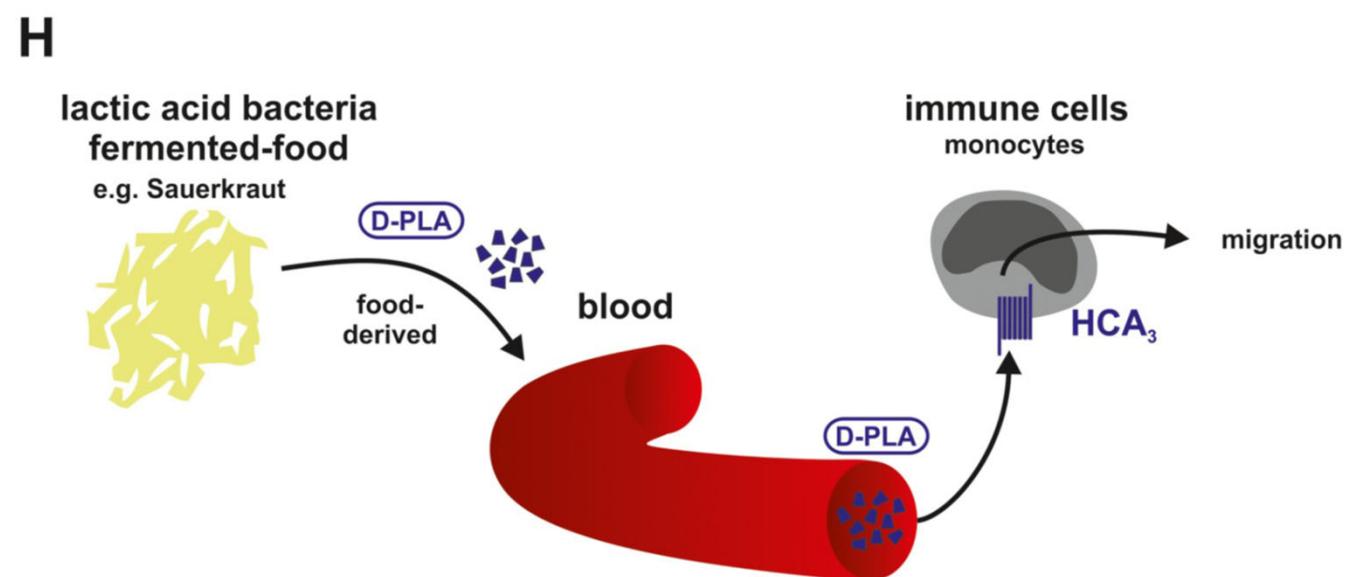
- Phenyllactic acid is a potent agonist of HCA3
 - Especially the D-form
- Produced by lactic acid bacteria
 - Sauerkraut rich in PLA. Intervention with Sauerkraut (6 g/kg bodyweight)
 - Clear PLA increase in both plasma and urine



How does fermented influence the immune system??

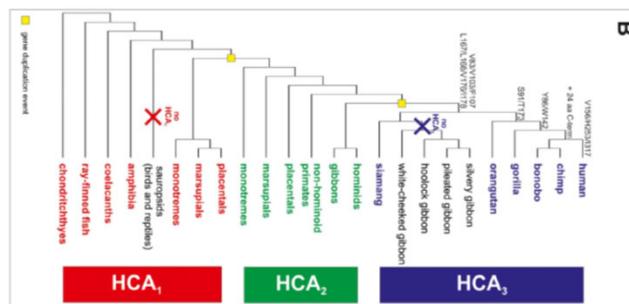
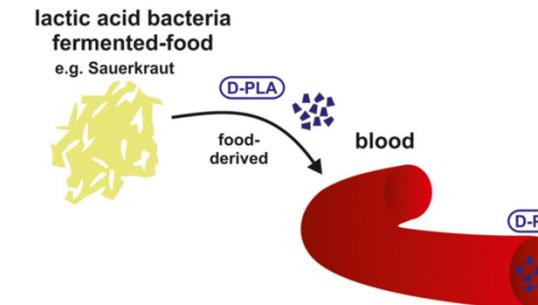


- Human peripheral blood mononuclear cells (PBMCs) exposed to agonists
- Reduced activation



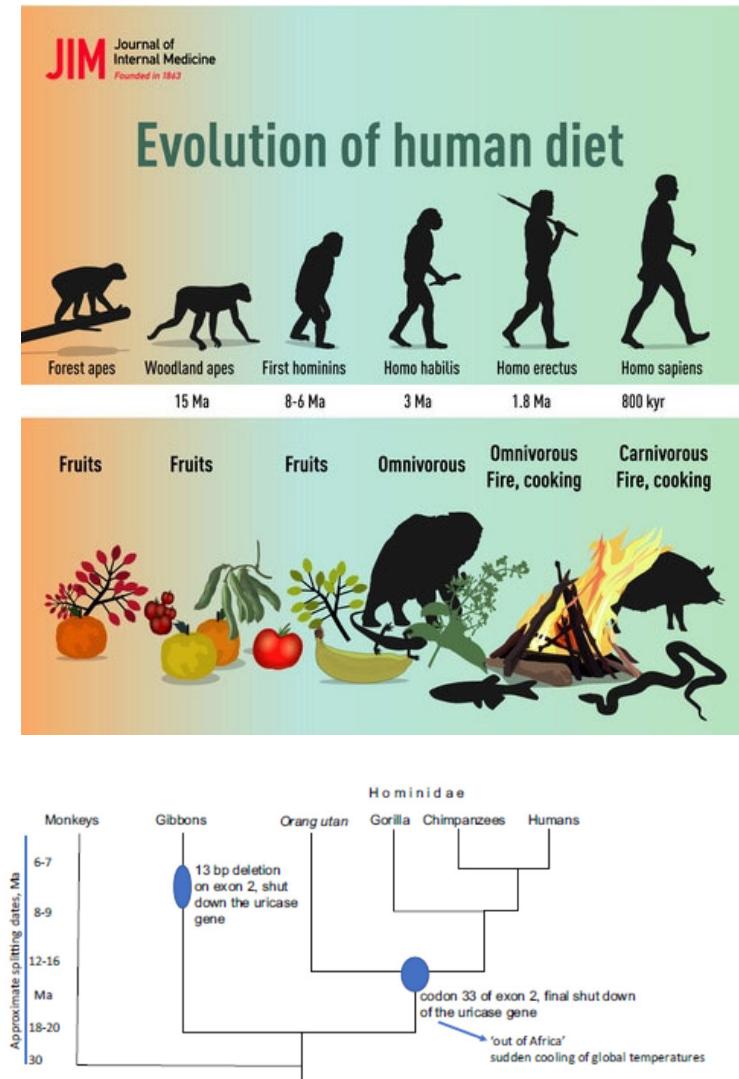
A hypothesis

H



We shape
food – but
perhaps
(fermented)
food also
shaped us?

Peters et al. (2019), PLoS Genetics 15: e1008145
Andrews, Johnson (2020), J Intern Med 287:226–237.



To sum up

- The gut microbiome plays an important role in health and disease
- Diet is the most important driver of GM development, composition and function
 - But also other environmental factors important
- Fermented products have effects beyond their macro-nutritional composition
 - There are specific receptors in humans (and a few very related species) that specifically sense these metabolites

Corynebacteria/Staphylococci

Henrik Siegumfeldt

KØBENHAVNS UNIVERSITET

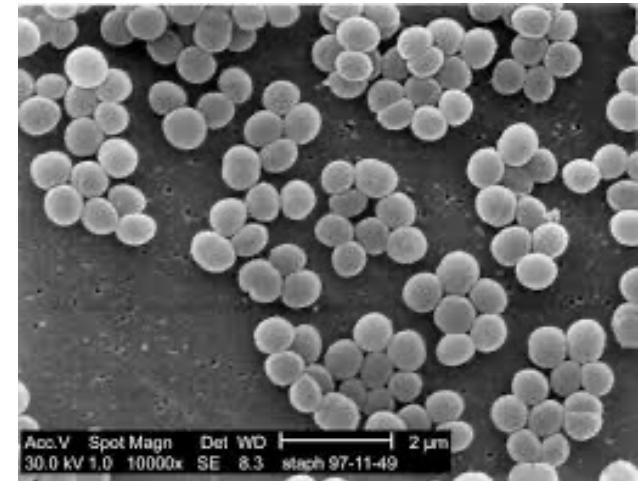


Staphylococcus & Corynebacterium in food fermentations

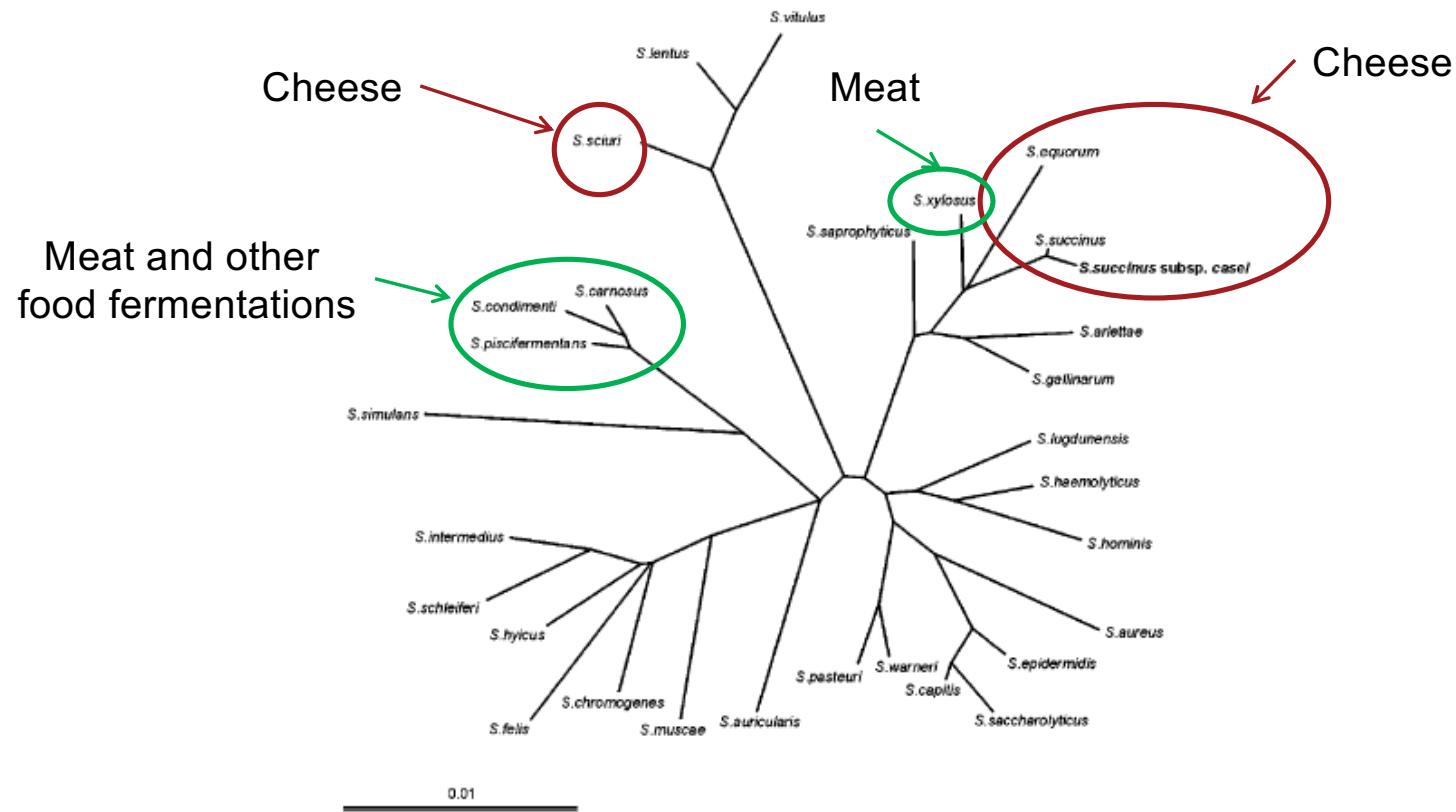
- Mediterranean-type of fermented sausages
 - *Staphylococcus*
 - Contribute to flavour
 - Contribute to colour
- Surface ripening in cheese
 - Red smear
 - Yeast (primarily *Debaryomyces hansenii*)
 - *Staphylococcus* spp. (5-15% abundance)
 - *Corynebacterium* spp. (2-70% abundance)
 - Contribute to flavour
 - Protects against mould and *Listeria* infections

Staphylococcus (Family *Staphylococcaceae*)

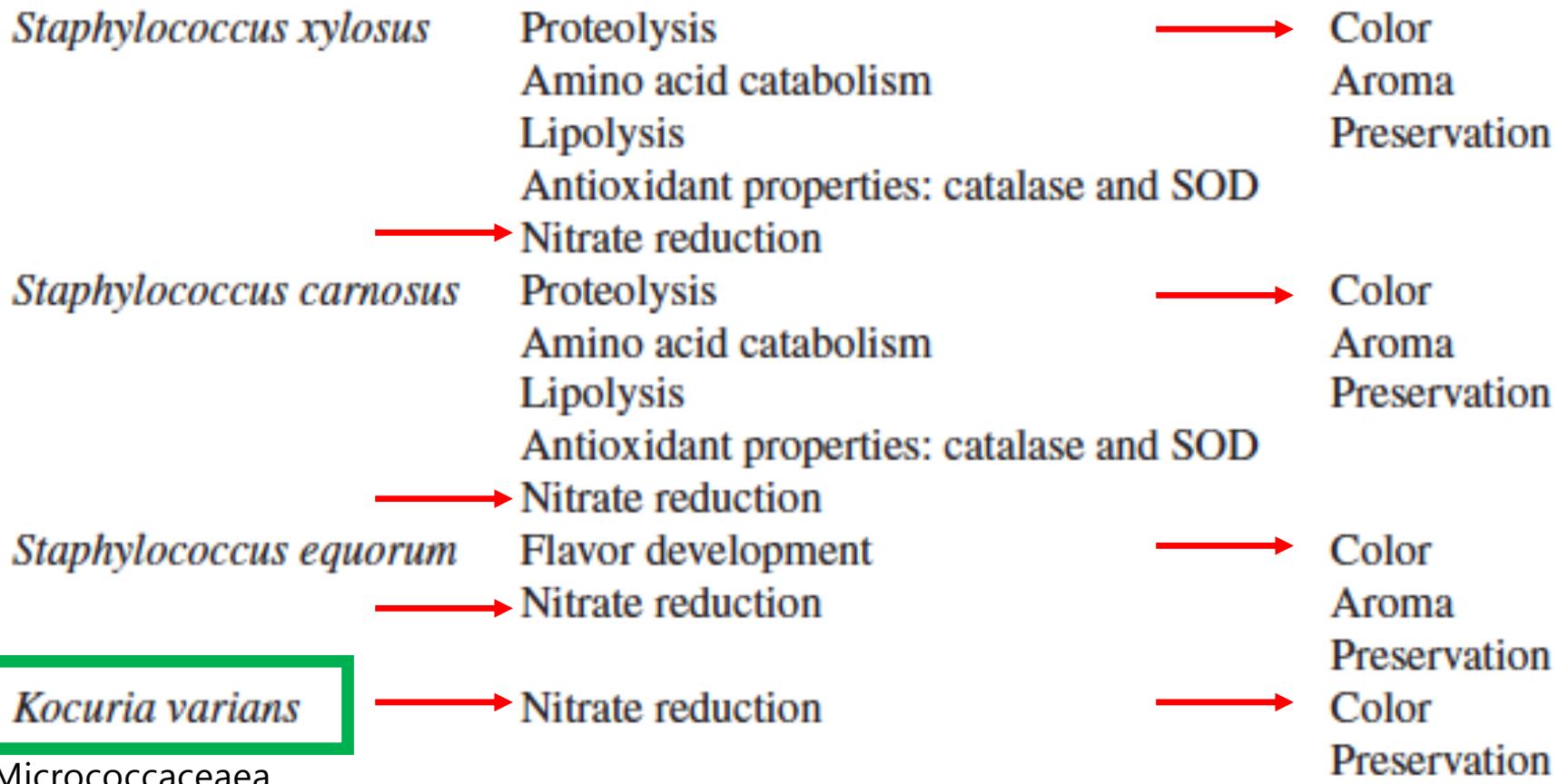
- At least 44 species (but new species and subspecies are constantly added)
- Gram-positive (Bacillota/Firmicutes) (=low G+C Gr+)
- Catalase positive (different from LAB)
- Round cocci in tetrads or irregular clusters
- Resistant to lysozyme
- Sensitive to lysostaphin (bacteriocin)
- Many are pathogens or opportunistic pathogens



Staphylococcus related to food fermentations



Role of *Staphylococcus* (and similar) in sausage fermentation



From P.S. Conconcelli & C. Fontana. Characteristics and applications of microbial starters in meat fermentations.
p. 129-148 in F. Toldrá (ed) Meat Biotechnology. Springer, 2008

NO₂⁻ conversion to NO and generation of red color

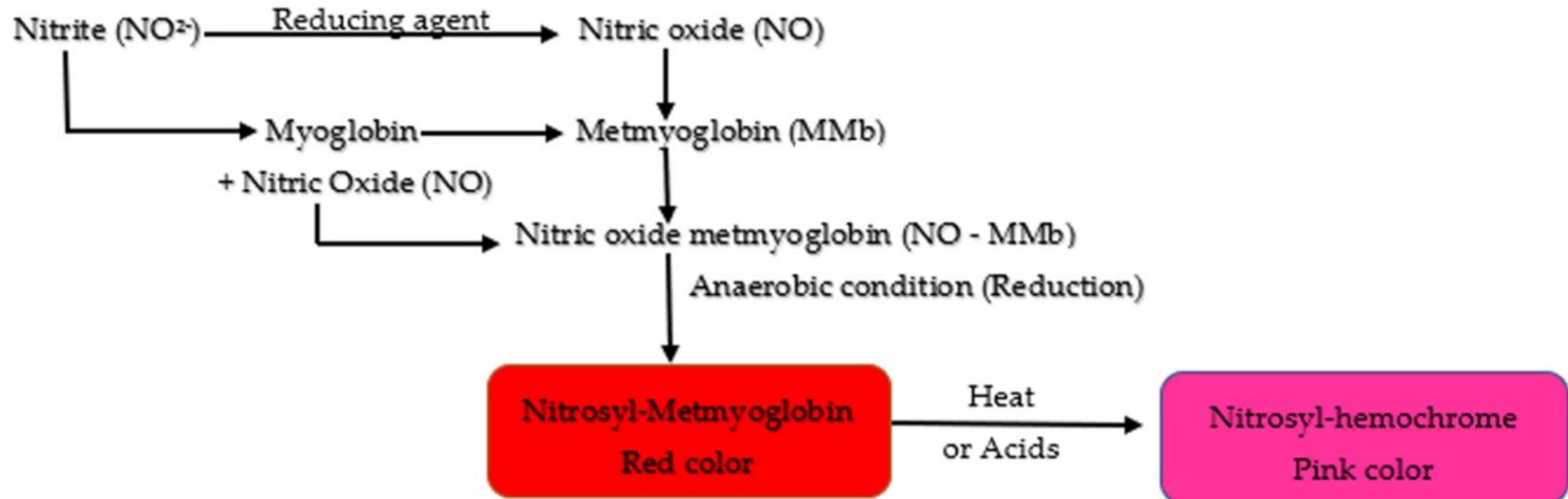


Figure 3. Mechanism of color development in cured meats.

Shakil, M.H.; Trisha, A.T.; Rahman, M.; Talukdar, S.; Kobun, R.; Huda, N.,; Zzaman, W. Nitrites in Cured Meats, Health Risk Issues, Alternatives to Nitrites: A Review. *Foods* 2022, 11, 3355. <https://doi.org/10.3390/foods11213355>

Reactions for *Staphylococcus* found in food fermentations and related organisms

Species	Coagulase	Novobiocin	Lysostaphin	Lysozyme	NH ₃ Arg	NO ₃ ⁻	Use
<i>St. aureus</i>	Positive	Sensitive	Sensitive	Resistant	Positive	Reduce	Pathogen
<i>St. carnosus</i> subsp. <i>carnosus</i>	Negative	Sensitive	Sensitive	Resistant	Positive	Reduce	Dry sausage
<i>St. carnosus</i> subsp. <i>utilis</i>	Negative	Sensitive	Sensitive	Resistant	Positive	Reduce	Seafood sauces
<i>St. condimenti</i>	Negative	ND	Sensitive	Resistant	Positive	Reduce	Soy Sauce
<i>St. equorum</i> subsp. <i>equorum</i>	Negative	Resistant	Sensitive	Resistant	Negative	Reduce	Skin isolate
<i>St. equorum</i> subsp. <i>linens</i>	Negative	Sensitive	Sensitive	Resistant	Negative	Reduce	Cheese smear
<i>St. fleurettii</i>	Negative	ND	Sensitive	Resistant	Negative	Reduce	Goat milk cheese
<i>St. piscifermentans</i>	Negative	Sensitive	Sensitive	Resistant	Positive	Reduce	Fermented fish
<i>St. sciuri</i>	Negative	Resistant	Sensitive	Resistant	Negative	Reduce	Cheese smear
<i>St. succinus</i> subsp. <i>succinus</i>	Negative	Resistant	Sensitive	Resistant	Negative	Negative	Amber
<i>St. succinus</i> subsp. <i>casei</i>	Negative	Resistant	Sensitive	Resistant	Negative	Reduce	Cheese smear
<i>St. xylosus</i>	Negative	Resistant	Sensitive	Resistant	Negative	Reduce	Sausage

Coagulase test video: <https://www.youtube.com/watch?v=yKzFS1BC1J0>

The lysostaphin tests (clinical - bacteriocin)

Susceptibility to lysostaphin is a rapid test method in differentiating staphylococci from micrococci. Other differentiation tests, such as glucose fermentation, bacitracin or furazolidone susceptibility tests require at least 24 hours incubation. In contrast, the lysostaphin disk test is a 2.5 hour test.

The peptidoglycan in the cell walls of most *Staphylococcus* contain an interpeptide bridge consisting of glycine-rich peptides. Lysostaphin is an endopeptidase that cleaves these peptide linkages, rendering the cells susceptible to osmotic lysis (lysostaphin susceptible) (2,4). The interpeptide bridge of *Micrococcus* does not contain glycine. Since the presence of glycine is essential for the action of lysostaphin, the *Micrococcus* cells are not affected and are lysostaphin resistant. (2,4)



Showing positive reaction (lysostaphin susceptible).

A 1mL aliquot of a heavy *Staphylococcus aureus* (ATCC ® 25923) suspension (McFarland 2.0) in saline was incubated aerobically for 2.5 hours at 35°C. with a Lysostaphin Differentiation Disk (Cat. no. Z112). The loss of turbidity was indicative of a positive reaction (lysostaphin susceptible), as compared to the negative control (LEFT).



Showing negative reaction (lysostaphin resistant).

A 1mL aliquot of a heavy *Micrococcus luteus* (ATCC ® 49732) suspension (McFarland 2.0) in saline was incubated aerobically for 2.5 hours at 35°C. with a Lysostaphin Differentiation Disk (Cat. no. Z112). No loss of turbidity was indicative of a negative reaction (lysostaphin resistant) as compared to the negative control (LEFT).

Novobiocin test (clinical - antibiotic)

The HardyDisk™ Novobiocin Differentiation Disks are useful in presumptively distinguishing *S. saprophyticus* from other CoNS. Other human staphylococcal species that are novobiocin-resistant (*S. cohnii*, *S. xylosus*, *S. pulvererii*) are rarely isolated from patients.⁽²⁻⁵⁾ The novobiocin susceptibility test can be done using a plate method (18-24 hour test) or a tube method (5 hour test). A study conducted by Harrington and Gaydos in 1984, concluded that the novobiocin tube test is an acceptable method when performed using Tryptic Soy Broth (TSB), 3ml, and has the advantage of taking only 5 hours.⁽⁶⁾ While the plate method is the more common approach of the two, both procedures are outlined in the section entitled "Procedure".



**Novobiocin-resistant
(zone of inhibition $\leq 16\text{mm}$).**

Staphylococcus saprophyticus (ATCC® 15305) growing around a HardyDisk™ Novobiocin Differentiation Disk (Cat. no. Z7291). Incubated aerobically on TSA (Cat. no. G60) for 24 hours at 35°C.



**Novobiocin-sensitive
(zone of inhibition $> 16\text{mm}$).**

Staphylococcus epidermidis (ATCC® 12228) inhibition zone around a HardyDisk™ Novobiocin Differentiation Disk (Cat. no. Z7291). Incubated aerobically on TSA (Cat. no. G60) for 24 hours at 35°C.

Red smear cheeses

- Danish cheeses
- Havarti
- Esrom
- Danbo
- European cheeses
- Limburger
- Tilsiter
- Münster
- Romadour
- Appenzeller
- Gruyère
- St. Poulin

Red smear technology

- Why smear?
 - Prevent mould development on the surface
 - Enhance flavour development and cheese diversity
 - Reduce the risk of pathogen growth (e.g. *Listeria monocytogenes* and *Staphylococcus aureus*)
- "Old-new" red smear technology
 - Traditional smearing technology
 - Use old smear to smear new cheeses
 - Usually very efficient smearing
 - Growth of yeast to 10^6 - 10^8 cfu/cm² within a few days
 - Growth of bacteria to 10^9 cfu/cm² within 5-7 days
- Alternatively use of "pure" starter cultures
 - May be efficient
 - Lower risk of contamination

Red smear yeast components

- Yeast
 - *Debaryomyces hansenii*
 - *Geotrichum candidum*
 - *Kluyveromyces marxianus*
 - *Candida krusei*
 - Primary role is to consume/assimilate lactate on surface and deaminate amino acids
 - **pH increase**
 - Allows subsequent growth of coryneform bacteria that are sensitive to the lower pH of fresh cheese
 - Produce B-vitamins (strain dependent)
 - Panthothenate (B5) (1-150 µg/ml)
 - Niacin (B3) (13-180 µg/ml)
 - Riboflavin (B2) (5-200 µg/ml)
 - Biotin (B7) (0-3 µg/ml, few strains produce biotin)

Corynebacterium in food fermentations

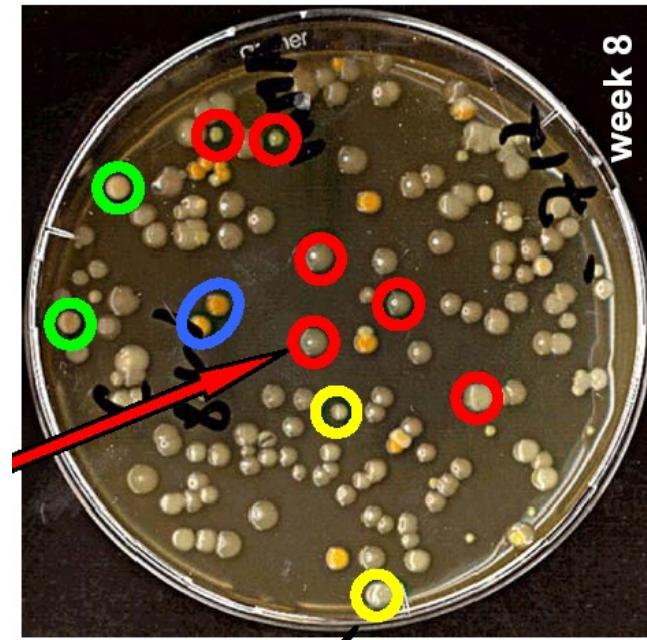
- Gram-positive (Actinomycetota/Actinobacteria) (=high G+C Gr+)
- Family
 - *Brevibacteriaceae*
 - *Brevibacterium* (33 species)
 - *Corynebacteriaceae*
 - *Corynebacterium* (125 species)
 - *Microbacteriaceae*
 - *Microbacterium* (117 species)
 - *Micrococcaceae*
 - *Arthrobacter* (95 species). Isolates from cheese have been transferred to *Glutamicibacter nicotianae* species

Red smear coryneform bacterial components

- Coryneform bacteria:
 - *Brevibacterium* species (33 species)
 - *B. linens*
 - *B. casei*
 - *Corynebacterium* species (125 species)
 - *C. ammoniagenes*
 - *C. casei*
 - *C. variabile*
 - *Microbacterium* species (117 species)
 - *M. lacticum*
 - *M. gubbeenense*

Propagation of smear microorganisms from cheese

- Yeasts
 - YGC agar (aerobic, 25C)
- Bacteria
 - Trypticase Soy Agar
 - Plate Count Agar
 - Modified Milk Agar
 - Aerobic
 - 25C
 - In light
 - Add 3-5 % salt



Effect of light on pigmentation of coryneform bacteria from cheese

Effect of Light on Pigmentation of Coryneform Bacteria from Different Cheese (from Mulder *et al.*¹⁶)

Organism	Source ^a	No. of strains examined	Colour of colonies grown in the	
			Dark	Light
Coryneform bacteria from cheese; grey-white strains	Ed, Go, Lei, Limb, Mesh	11	Grey-white	Grey-white; growth reduction in 6 strains
	Go, Mesh	6	Grey-white	Light yellow; growth reduction in 2 strains
	Go, Limb, Mesh	6	Grey-white with pink shade	Grey-white with pink shade; growth reduction in 4 strains
<i>Br. linens</i> ^(3,5,6,7,8,9)	Culture collections	6	Orange	Orange
<i>Br. linens</i> ^(1,2)		2	Light cream-yellow	Orange
Orange-pigmented strains	Ed, Go, Ke, Limb, Mam, Mesh, Pe, Rom, St.P	13	Orange	Orange
	Ed, Go, He, Ho, Mam, Marv, Mesh, Mu, Rom, St.P, Vach	16	White	Orange; growth reduction in 1 strain
	Go, Ke	3	Light cream-yellow	Orange

^a Cheese of the type: Ed, Edam; Go, Gouda; He, Hervse; Ho, Hohenheim; Ke, Kernhemmer; Lei, Leidse kanter; Limb, Limburger; Mam, Mamirolle; Marv, Marville; Mesh, Meshanger; Mu, Munster; Pe, Pénitent; Rom, Romadour; St.P, St. Paulin; Vach, Vacherin Mont d'Or. (Reproduced with permission of *Journal of Applied Bacteriology*.)

Differentiation of Coryneform bacteria

Table 1. Differentiation of *Brevibacterium* from other actinomycete and coryneform genera.

Genus	Morphology	Wall diamino acid	Arabinogalactan polymer	Mycolic acids	Fatty acid type ^a	Major menaquinone
<i>Brevibacterium</i>	Marked rod-coccus cycle	meso-A ₂ pm	–	–	S,A,I	MK-8(H ₂)
<i>Arthrobacter</i>	Marked rod-coccus cycle	Lysine	–	–	S,A,I	MK-9(H ₂) or MK-8/MK-9
<i>Cellulomonas</i>	Irregular rods, some coccoid forms	L-Ornithine	–	–	S,A,I	MK-9(H ₄)
<i>Clavibacter</i>	Irregular rods	Diaminobutyric acid	–	–	S,A,I	MK-9, MK-10
<i>Corynebacterium</i>	Irregular rods	meso-A ₂ pm	+	+	S,U,(T)	MK-8(H ₂), MK-9(H ₂)
<i>Curtobacterium</i>	Irregular rods	D-Ornithine	–	–	S,A,I	MK-9
<i>Gordona</i>	Rod-coccus cycle	meso-A ₂ pm	+	+	S,U,T	MK-9(H ₂)
<i>Microbacterium</i>	Irregular rods, some coccoid forms	Lysine	–	–	S,A,I	MK-11, MK-12
<i>Rhodococcus</i>	Rods and coccoid forms	meso-A ₂ pm	+	+	S,U,T	MK-8(H ₂)

+, present; –, absent.

^aS, straight-chain saturated; A, anteiso; I, iso; T, tuberculostearic acid; (), variable.

Molecular techniques used to identify coryneform bacteria and staphylococci at species and strain level

- Species level:
 - 16S rRNA sequencing (with some exceptions)
 - rep-PCR
- Strain level
 - PFGE
 - rep-PCR
 - Whole Genome Sequencing

Brevibacterium

- Gram-positive (young) or gram-variable (older)
 - Short rod with clear rod-cocci cycle
 - Non-spore former
 - Catalase positive
 - Non-motile
 - Obligatory aerobic
 - Respiratory metabolism
- 33 species / 2 in red smear:
- *B. linens*
 - *B. casei*
- 16S rDNA sequences vary quite much for *B. linens* (97-100 % similarity among strains)
- Probably necessary to reevaluate taxonomy within *B. linens*

Differential characteristics of *Brevibacterium*

Table 2. Differential characteristics of species of the genus *Brevibacterium*.

Characteristic	<i>B. linens</i>	<i>B. casei</i>	<i>B. epidermidis</i>	<i>B. iodinum</i>
Colony color	Yellow-orange	Gray-white	Gray-white	Gray-white
Crystals of iodinin	—	—	—	+
Oxidase	—	—	—	+
Survival at 60°C for 30 min	—	+	+	—
GC content (mol%)	60–64	66–67	63–64	61–63

+, present; —, absent.

Brevibacterium linens: Pigmentation increases in light
 Pigment turns to pink colour with KOH

Arthrobacter

- Gram-positive (young) or gram-variable (older)
- Rods with clear rod-cocci cycle
- Non-sporeformer
- Non-motile or motile
- Catalase positive
- Obligatory aerobic
- Respiratory metabolism
- Usually yellow colonies from smear cheese
- 51 species/1 in red smear:
 - *A. nicotinae*
 - Now: *Glutamicibacter nicotianae*



Antimicrobials and bioprotective cultures

Henriette Lyng Røder
Associate Professor

Section for Food Microbiology, Gut Health and Fermentation
Department of Food Science
University of Copenhagen

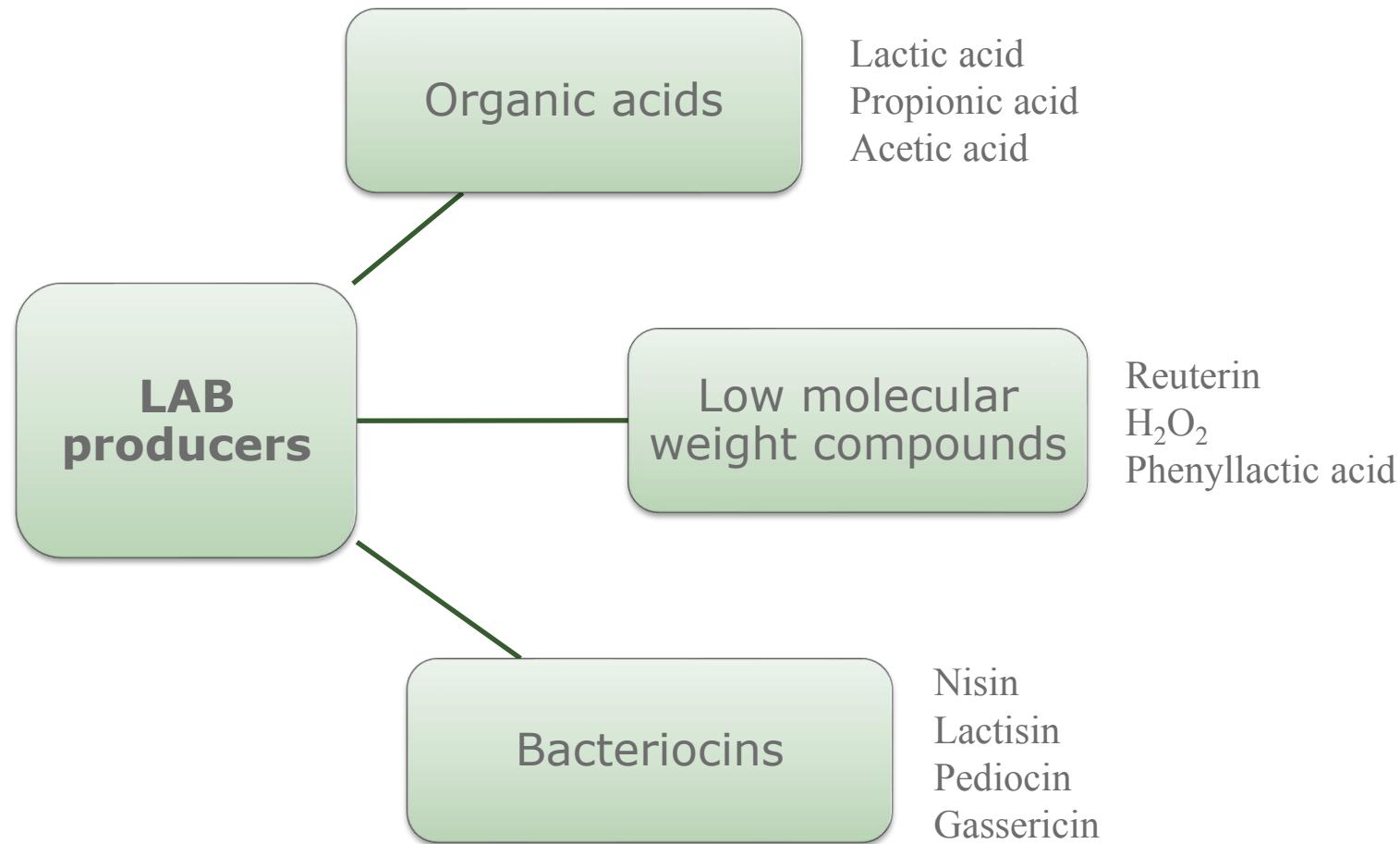


Agenda

- 1. Bacteriocins** are natural antimicrobial compounds produced by bacteria to inhibit or kill other bacterial strains.
- 2. Mechanisms** such as pore formation, nucleic acid degradation, and enzymatic actions are employed by bacteriocins to disrupt the growth of target bacteria.
- 3. Classification** divides bacteriocins into categories like lantibiotics and non-lantibiotics, each with unique properties.
- 4. Detection methods** are essential to identify the presence of bacteriocins and assess their effectiveness in food preservation.
- 5. Applications** in food preservation leverage bacteriocins' specificity to extend the shelf life of products while enhancing food safety.



Biopreservation: Inhibitor compounds produced by LAB



What are bacteriocins?

- **Peptides**
 - Sensitive to proteases, e.g. Proteinase K
- **Ribosomally synthesized**
- **Chromosomal or plasmid encoded in LAB**
 - Sakacin P (*Latilactobacillus sakei*)
 - *Chromosomal*
 - Nisin A (*Lactococcus. lactis*)
 - *Chromosomal* (conjugative transposon)
 - Pediocin PA-1 (*Pediococcus acidilactici*)
 - *Plasmid*
- **Hydrophobic nature**
- **Many are membrane-active peptides**

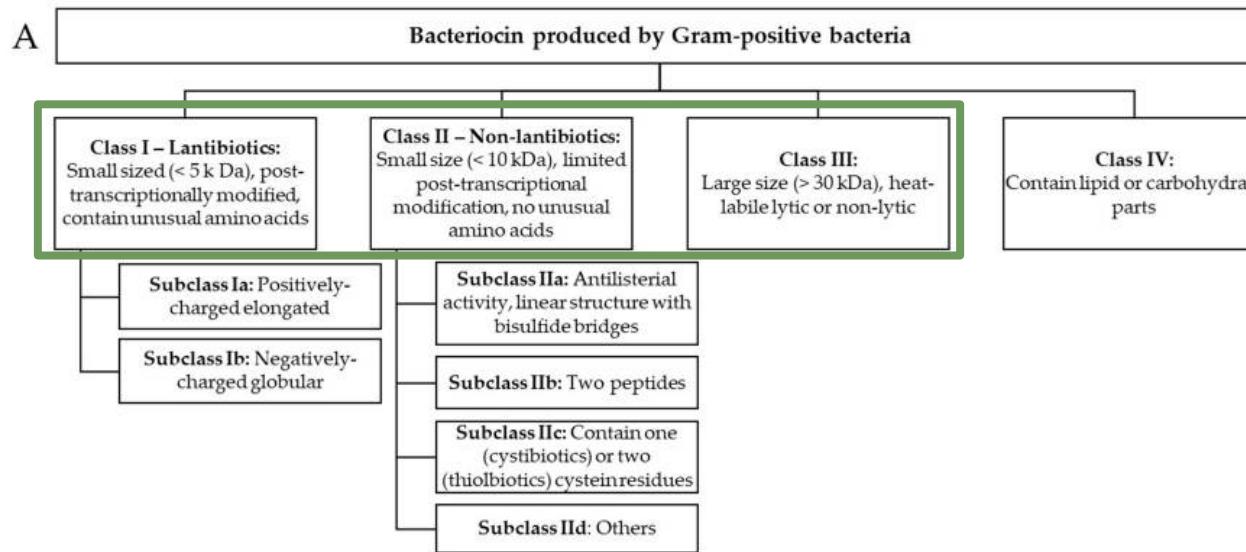


Bacteriocins

- Produced during late exponential growth phase
- Mainly secreted to the media/fermentation product
- Activity:
 - Bactericidal, bacteristatic, or sporcidal
- Active against:
 - Taxonomically related strains
 - E.g. other closely related LAB
 - Taxonomically unrelated species
 - E.g. *Bacillus* spp., *Clostridium* spp., *Listeria*, i.e. gram-positive bacteria



Schema of bacteriocin classification



Simons, A., Alhanout, K., & Duval, R. E.
(2020). *Microorganisms*, 8(5), 639.

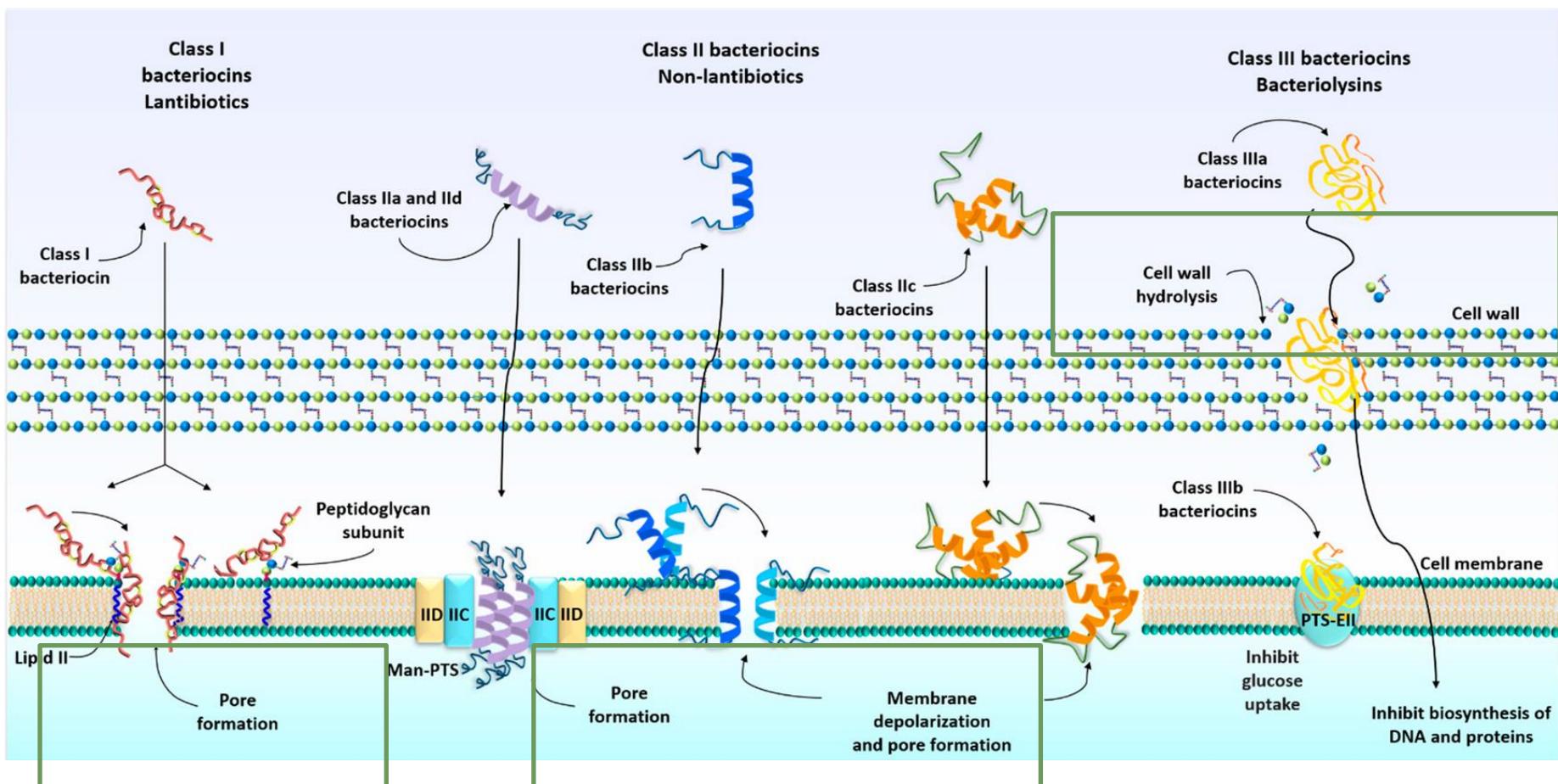


Classes of LAB bacteriocins

- **Class I:** Lantibiotics (< 5 kDa), Small heat-stable
 - A: Membrane active lantibiotics (e.g. nisin, lacticin 3147)
 - B: Inhibition of enzymes (e.g. mersacidin (*B. subtilis*))
- **Class II:** (< 10 kDa), Small heat-stable non-lantibiotics containing membrane active peptides
 - IIa : Pediocin-like bacteriocins (*Listeria*, active)
 - IIb : Two-peptide bacteriocins
 - IIc : Cyclic polypeptides
 - IID : Other peptide bacteriocins
- **Class III:** (>30 kDa), Large heat-labile bacteriocins
- **Class IV:** Complex, one/more chemical moieties (lipid/carbohydrate)



Bacteriocins: general mode of actions for class I-III bacteriocins



Hernández-González, J. C., et al. (2021).
Animals, 11(4), 979.



Bacteriocin examples

Table 2. Bacteriocins used in food preservation and their characteristics.

Bacteriocin class	Bacteriocin	Desirable properties	Producer organism	Target food contaminant	Type of food matrix preserved	Reference
Class Ia	Nisin	Heat stable at 121 °C for prolonged heating at pH 2. Become less heat stable at pH 5–7. Sensitive towards α-chymotrypsin, resistant to trypsin, elastase, carboxypeptidase A, pepsin, and erepsin.	<i>L. lactis</i> subsp. <i>lactis</i>	<i>Streptococcus thermophilus</i> , <i>Lactobacillus</i> spp., <i>L. monocytogenes</i> , <i>L. lactis</i> , <i>S. aureus</i> , <i>Clostridium botulinum</i> , <i>Bacillus cereus</i>	Processed cheese products, ricotta cheese, pasteurized milk and milk products, canned products, salad dressings, crumpets, sausages, meat products	Meghrouss, Lacroix, and Simard (1999)
Class Ic	Lacticin 3147A	Heat stable at 100 °C for 10 min at pH 5 or 90° C for 10 min at pH 7. Stable at room and low temperature, heat stable at 100° C for 60 min or 121° C for 10 min. Most stable at acid and neutral pH.	<i>L. lactis</i> DPC3147	<i>B. subtilis</i> , <i>S. aureus</i> , <i>L. monocytogenes</i> <i>Lactobacillus fermentum</i>	Infant formula, yoghurt, cottage cheese, sausages, ground beef	Ryan et al. (1999)
	Plantaricin C	Resistant to pepsin, proteinase K, α-amylase, and lipase.	<i>L. plantarum</i>	<i>Clostridium</i> , <i>Bacillus</i> , <i>Staphylococcus</i> and also to <i>Listeria monocytogenes</i>	Dairy products, meat, canned foods	Gonzalez et al. (1996)
Class IIa	Pediocin PA-1	Stable at pH 4 to 6, becomes less stable as pH increases. Heat stable at 80° C for 60 min or 100° C for 10 min. Resistant to phospholipase C, catalase, lysozyme, DNases, RNAses, and lipase.	<i>Pediococcus acidilactici</i>	<i>Lactobacillus helveticus</i> , <i>Pediococcus pentosaceus</i> , <i>L. monocytogenes</i>	Cheddar cheese, munster cheese, liquid whole egg, meat products (sausages, meat sticks),	Rodriguez, Martinez, and Kok (2002)
Class IIb	Enterocin 1071	The peptides are heat resistant (100° C, 60 min; 50% of activity remained after 15 min at 121°C), remain active after 30 min of incubation at pH 3 to 12, and are sensitive to treatment with proteolytic enzymes.	<i>Enterococcus faecalis</i> BFE 1071	<i>L. monocytogenes</i> , <i>Listeria innocua</i> , <i>Enterococcus faecalis</i> , <i>Enterococcus faecium</i>	Fish spread	Balla et al. (2000)
Class IIc	Enterocin EJ97	Very stable under mild heat conditions and is sensitive to proteolytic enzymes	<i>E. faecalis</i> EJ97	<i>Bacillus</i> spp., <i>E. faecalis</i> , <i>L. monocytogenes</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>Geobacillus stearothermophilus</i>	Canned vegetable foods and drinks	Galvez et al. (1998)
Class IV	Enterocin AS-48	Active at pH 9.0 and in combination with moderate heat treatment. Inactivated by heated for 5 min at 65°C in an alkaline (pH 9.0). Compatible with several chemical compounds: EDTA, lactic acid, peracetic acid, polyphosphoric acid, sodium hypochlorite, hexadecylpyridinium chloride, propyl-p-hydroxybenzoate, and hydrocinnamic acid	<i>Enterococcus faecalis</i> subsp. <i>liquefaciens</i> S-48	<i>Bacillus</i> spp., <i>S. aureus</i> , <i>L. monocytogenes</i> , <i>E. faecalis</i> , <i>E. faecium</i> , <i>E. coli</i> <i>Alicyclobacillus acidoterrestris</i> , <i>Bacillus</i> spp., <i>Paenibacillus</i> spp., <i>Geobacillus stearothermophilus</i> , <i>Brochotrix thermophila</i> , <i>Staphylococcus carnosus</i> , <i>Lactobacillus sakei</i> and other spoilage lactic acid bacteria	Ready-to-eat salad, model sausages, canned fruits and vegetables, raw fruits and fruit juices, vegetable sauces, vegetable soups, and purees	Cobo Molinos et al. (2008)

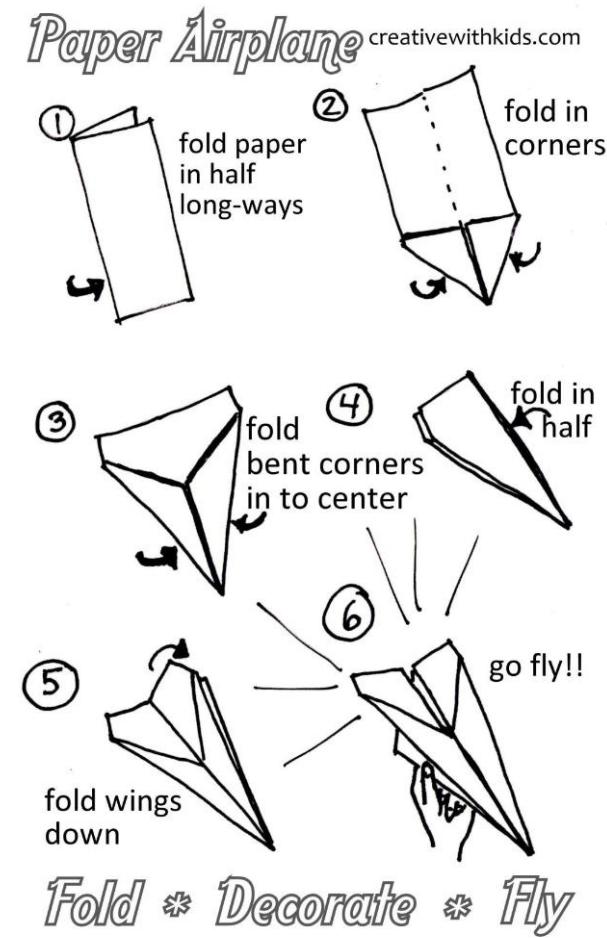
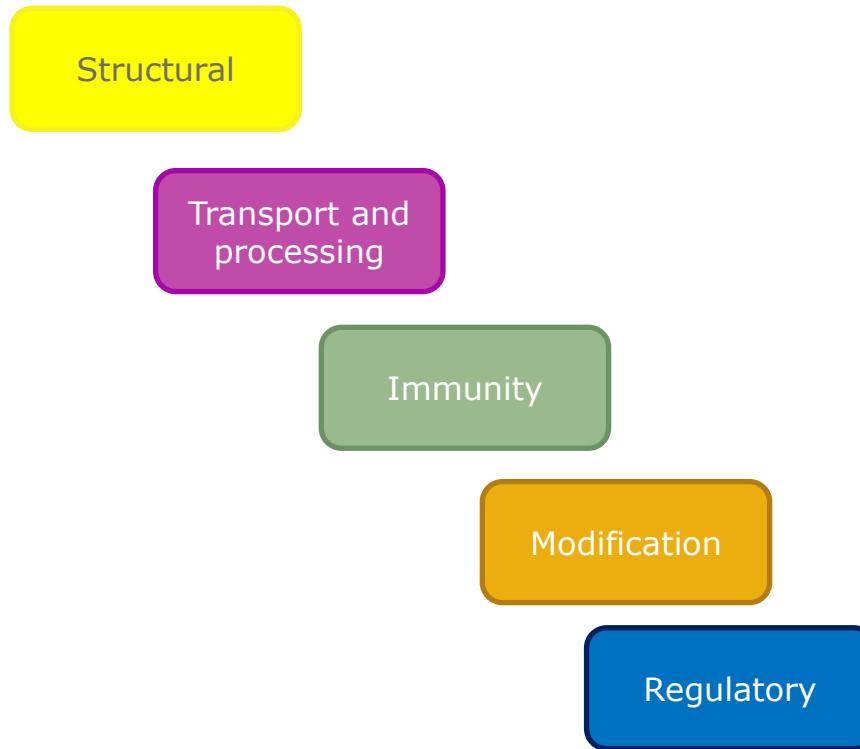


Classes of LAB bacteriocins

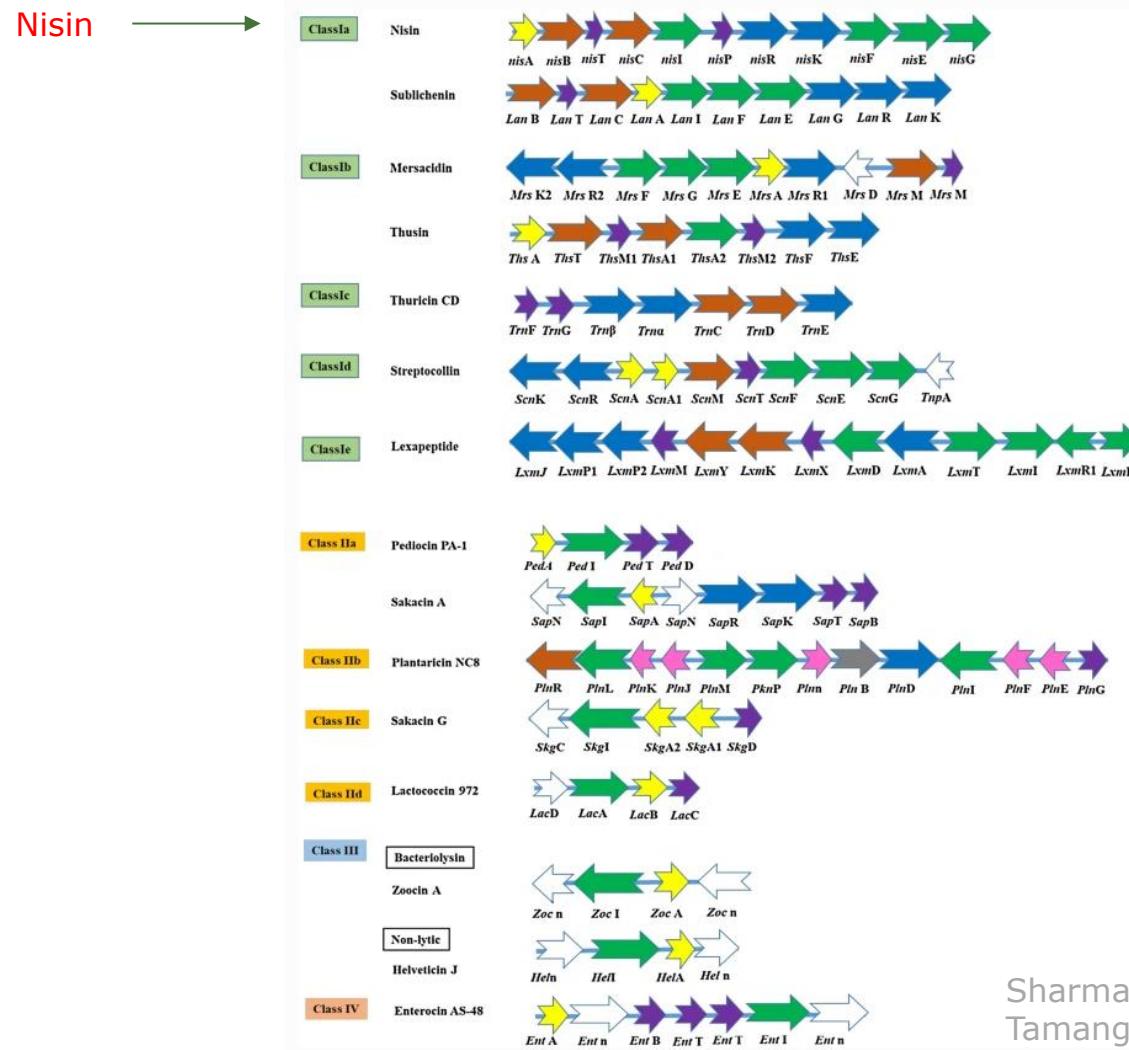
- **Class I:** Lantibiotics (< 5 kDa), Small heat-stable
 - A: Membrane active lantibiotics (e.g. nisin, lacticin 3147)
 - B: Inhibition of enzymes (e.g. mersacidin (*B. subtilis*))
- **Class II:** (< 10 kDa), Small heat-stable non-lantibiotics containing membrane active peptides
 - IIa : Pediocin-like bacteriocins (*Listeria*, active)
 - IIb : Two-peptide bacteriocins
 - IIc : Cyclic polypeptides
 - IID : Other peptide bacteriocins
- **Class III:** (>30 kDa), Large heat-labile bacteriocins
- **Class IV:** Complex, one/more chemical moieties (lipid/carbohydrate)



Gene cluster responsible for production and functionality of nisin



Schematic representation of gene clusters



Orange arrows = modification

Purple arrows = transport and processing

Green arrows = immunity

Blue arrows = regulatory

Yellow arrows = structural

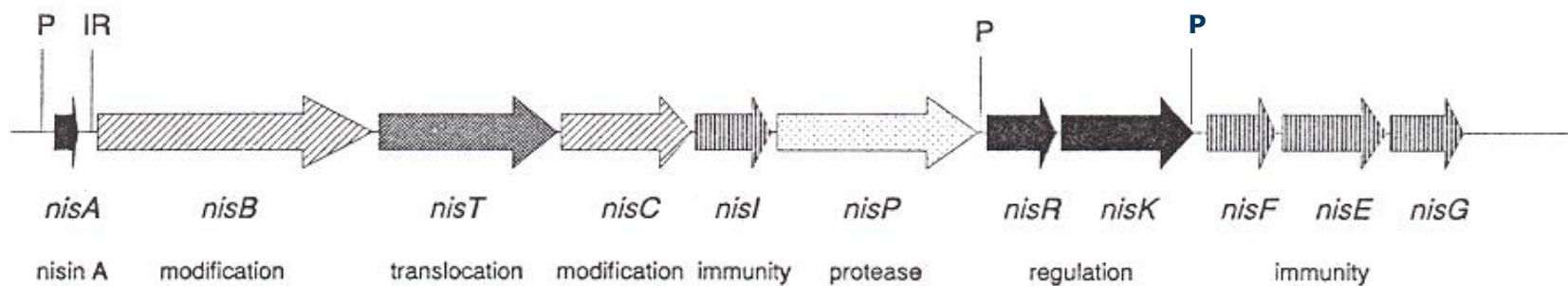
White and pink = unknown function

Sharma, B. R., Halami, P. M., & Tamang, J. P. (2021). *Food Science and Biotechnology*, 1-16.



Genetic structure of the *nisA*-operon

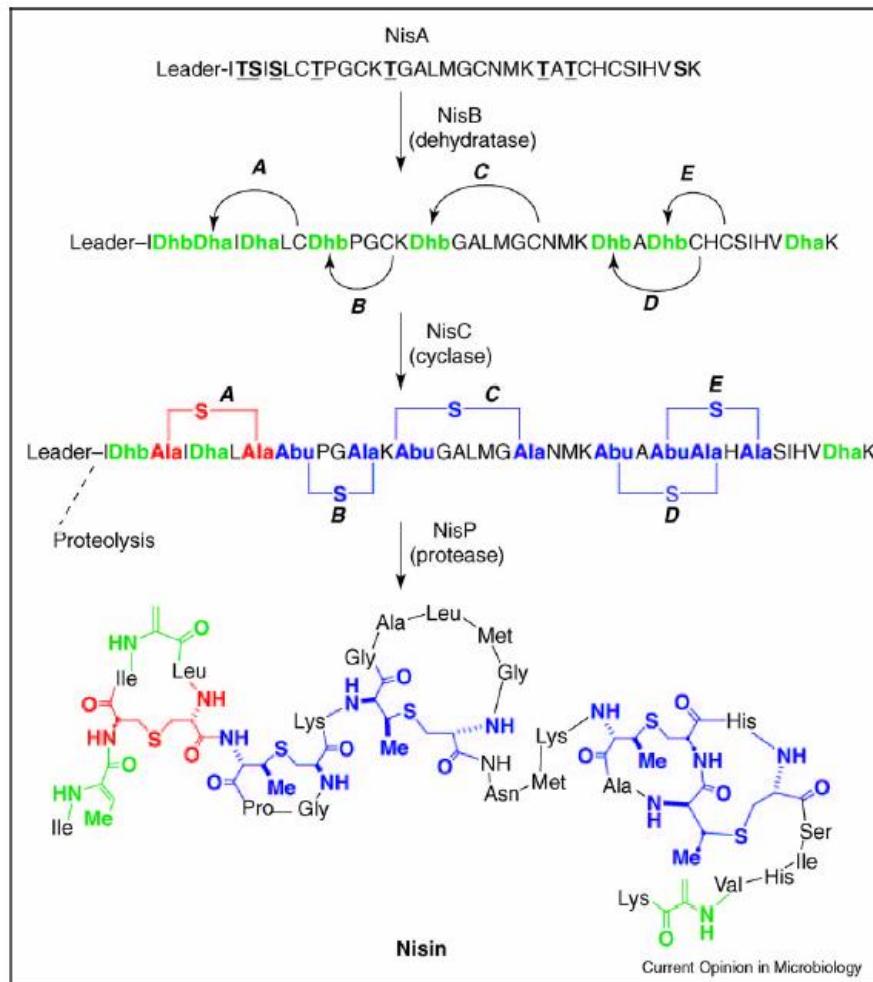
- The *nisA*-operon is located on a 70 kilo base pairs conjugative transposon usually also encoding genes for sucrose utilization
- The *nisA*-operon consists of 11 genes



Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G., Luesink, E. J., & de Vos, W. M. (1995). *Journal of Biological Chemistry*, 270(45), 27299-27304.



Maturation of Nisin A



- Nisin A is synthesized as a 57 aa pre-Nisin A
- NisB and NisC post-translationally modify pre-Nisin A
- NisT transports pre-Nisin A out of the cell where NisP cleaves off the leader peptide (23 aa)

Adapted from: Patton, G. C., & Van Der Donk, W. A. (2005). *Current opinion in microbiology*, 8(5), 543-551.



Mechanisms, nisin and other LAB bacteriocins

Two steps (within few minutes):

1. Adsorption (reversible)

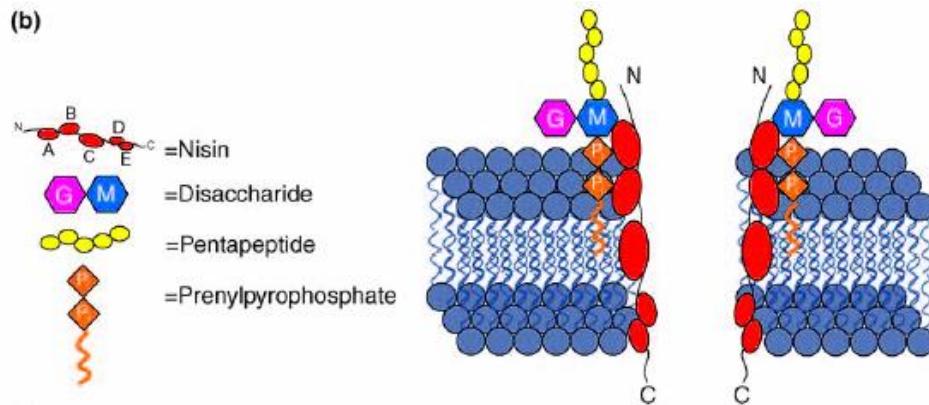
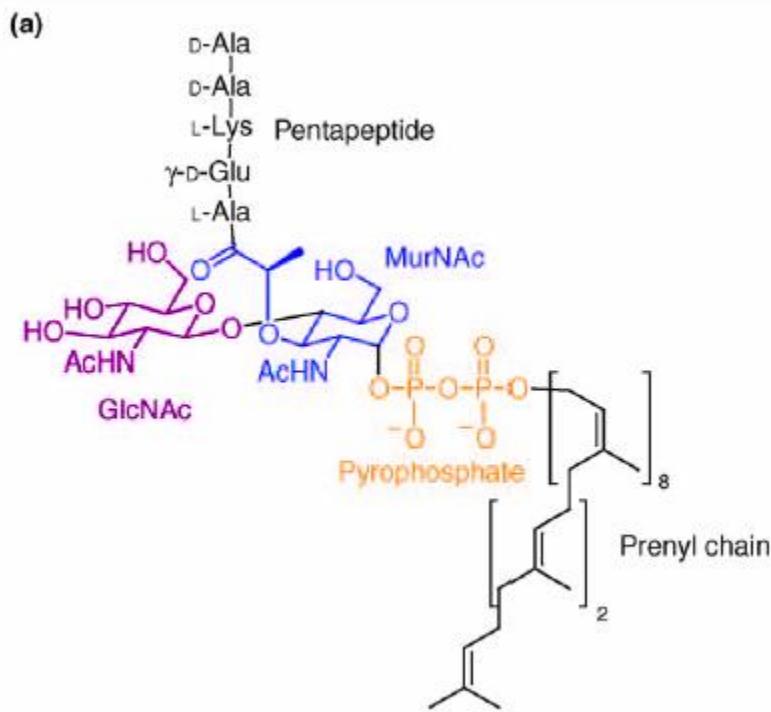
- Specific (receptors on the target cell surface, i.e. docking molecule)
- Non-specific (maybe because we did not recognize the docking molecule)

2. Action (irreversible)

- Creation of pores in the membrane
 - Increased membrane permeability
 - Efflux of cytoplasmic constituents, e.g. K⁺ ions, ATP, proteins, nucleic acids.
 - Dissipation of the PMF
 - No energy for cellular metabolism → growth inhibition → death



Nisin mode of action

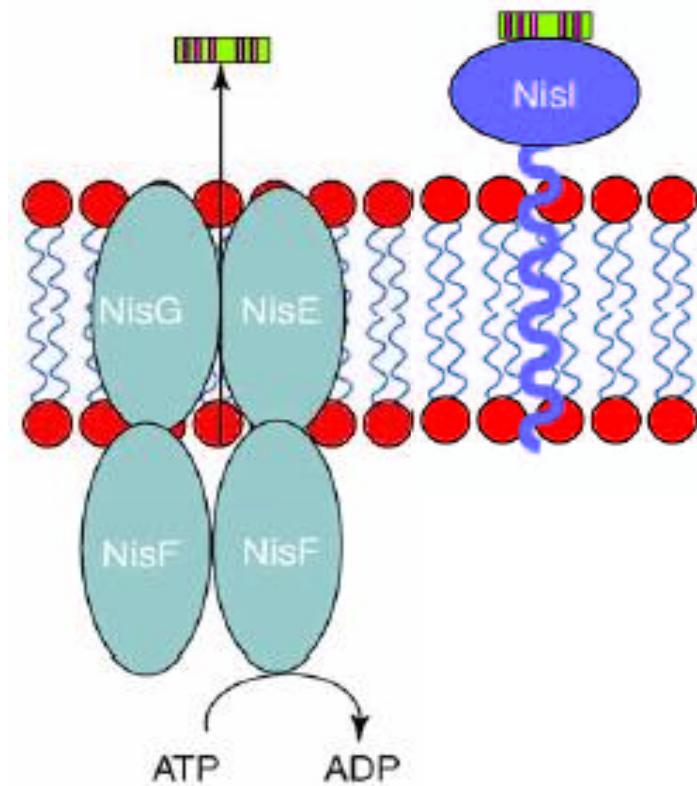


- Nisin recognizes Lipid II

Adapted from: Patton, G. C., & Van Der Donk, W. A. (2005). *Current opinion in microbiology*, 8(5), 543-551.



Nisin immunity mechanisms

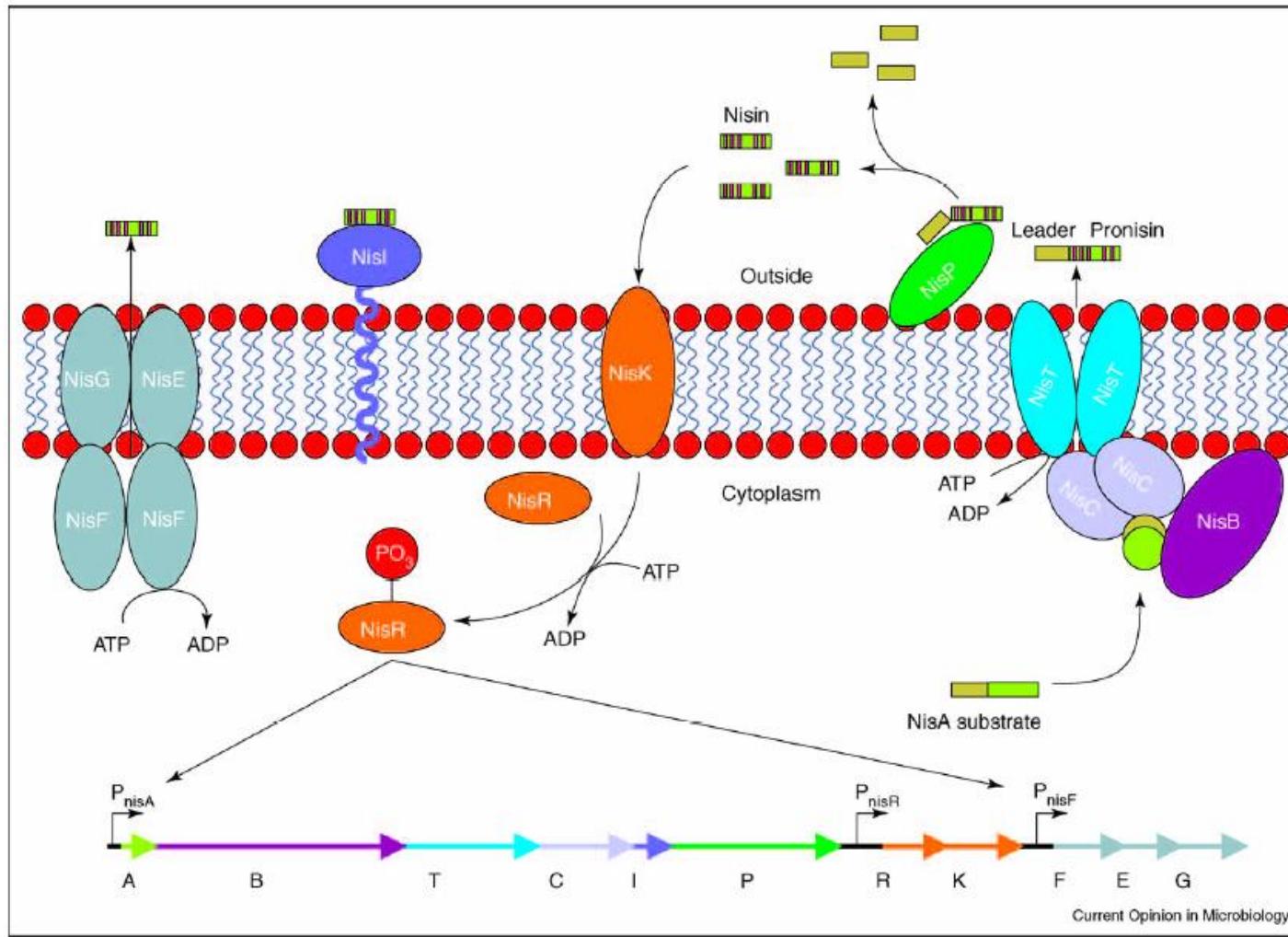


- NisI and NisEFG are involved in immunity
- NisI sequester nisin i.e. lower the concentration near the membrane
- NisEFG uses energy to “pump” nisin away from membrane

Adapted from: Patton, G. C., & Van Der Donk, W. A. (2005). *Current opinion in microbiology*, 8(5), 543-551.



Regulation of nisin production



Adapted from: Patton, G. C., & Van Der Donk, W. A. (2005). *Current opinion in microbiology*, 8(5), 543-551.



Natural and bioengineered variants of nisin

	Unmodified amino acid sequences	Origin
Natural variants		
Nisin A	ITSISLCTPGCKTGALMGC NMKT TATCHCSIHVSK	<i>Lactococcus lactis</i> strains (Gross and Morell 1971)
Nisin Z	ITSISLCTPGCKTGALMGC NMKT TATCNCISIHVSK	<i>Lc. lactis</i> NIZO 22186 (Mulders et al. 1991)
Nisin F	ITSISLCTPGCKTGALMGC NMKT TATCNC S HVS K	<i>Lc. lactis</i> subsp. <i>lactis</i> F10 (De Kwaadsteniet et al. 2008)
Nisin Q	ITSISLCTPGCKTG V LMGC NLKT TATCNC S HVS K	<i>Lc. lactis</i> 61-14 (Zendo et al. 2003)
Nisin H	FTS ISMCTPGCKTGALMT CNYK TATCHCSIKV S	<i>Streptococcus hyointestinalis</i> (O'Connor et al. 2015)
Nisin U	ITS K SLCTPGCKTGILMT CPLK TATCG CHFG	<i>Streptococcus uberis</i> (Wirawan et al. 2006)
Nisin U2	VTS KS L CTPGCKTGILMT CPLK TATC G CHFG	<i>Strep. uberis</i> (Wirawan et al. 2006)
Nisin P	VTS KS L CTPGCKTGILMT CAIK TATCG CHFG	<i>Streptococcus gallopticus</i> subsp. <i>pasteurianus</i> (Zhang et al. 2012)
Bioengineered variants		
Nisin A S29A	ITSISLCTPGCKTGALMGC NMKT TATCH CAI HVS K	<i>Lc. lactis</i> NZ9800 (Field et al. 2012)
Nisin A S29D	ITSISLCTPGCKTGALMGC NMKT TATCH CD IHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2012)
Nisin A S29E	ITSISLCTPGCKTGALMGC NMKT TATCH CHE IHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2012)
Nisin A S29G	ITSISLCTPGCKTGALMGC NMKT TATCH CGI HVS K	<i>Lc. lactis</i> NZ9800 (Field et al. 2008)
Nisin A K22T	ITSISLCTPGCKTGALMGC NM TATCHCSIHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2008)
Nisin A N20P	ITSISLCTPGCKTGALMGC P MKTATCHCSIHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2008)
Nisin A M21V	ITSISLCTPGCKTGALMGC NV KTATCHCSIHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2008)
Nisin A K22S	ITSISLCTPGCKTGALMGC NM STATCHCSIHV S	<i>Lc. lactis</i> NZ9800 (Field et al. 2008)
Nisin Z N20K	ITSISLCTPGCKTGALMGC KM KTATC N CSIHV S	<i>Lc. lactis</i> NZ9800 (Yuan et al. 2004)
Nisin Z M21K	ITSISLCTPGCKTGALMGC NK KTATC N CSIHV S	<i>Lc. lactis</i> NZ9800 (Yuan et al. 2004)

Amino acids in blue letters indicate the flexible hinge region. Yellow highlights indicate amino acid substitutions compared to nisin A. Please note that this table does not contain all variants that have been reported to date.

Shin, J. M., Gwak, J. W., Kamarajan, P., Fenno, J. C., Rickard, A. H., & Kapila, Y. L. (2016). *Journal of applied microbiology*, 120(6), 1449-1465.

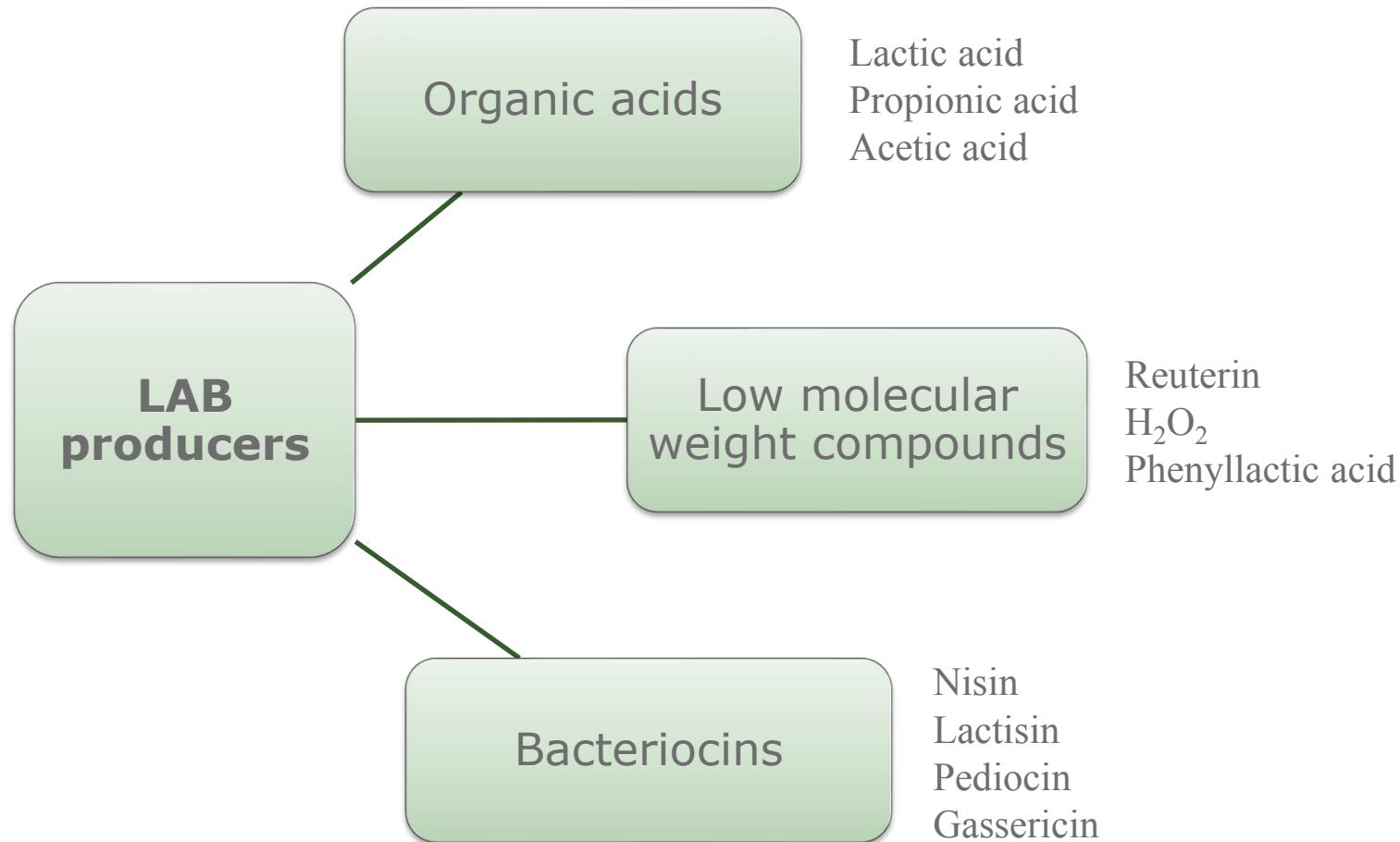


Bacteriocin detection methods

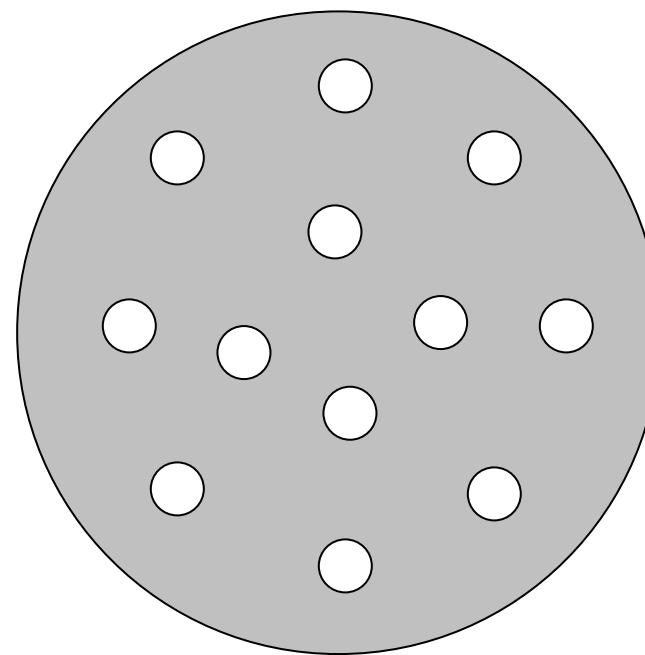
- Colony inhibition zone assay
- Agar well diffusion assay
 - Diameter of inhibition zone
- Microtiter growth inhibition assay
 - MIC ~ Minimal Inhibitory Concentration
- Immuno-assay
- Sequencing



Biopreservation: Inhibitor compounds produced by LAB



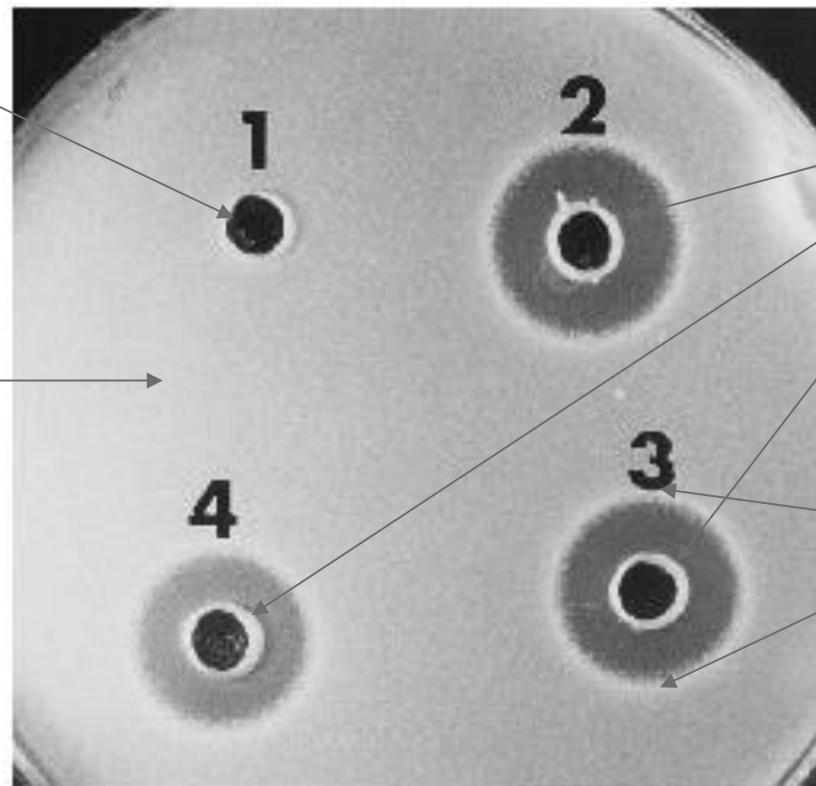
Determination of Inhibition by Agar Well Diffusion Assay (ADA)



The agar well diffusion assay (ADA)

Well containing supernatants not inhibitory to the indicator bacteria

Lawn of indicator bacteria



Wells containing supernatants inhibitory to the indicator bacteria

Inhibition zone diameter

Ryan, M. P., Rea, M. C., Hill, C., & Ross, R. P. (1996). *Applied and environmental microbiology*, 62(2), 612-619.

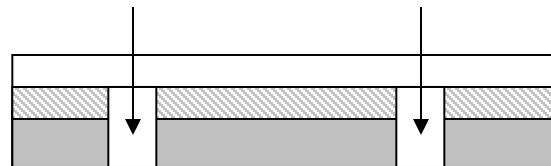
Antimicrobial compounds from LAB

- Organic acids
 - Lactic acid, acetic acid
- Low molecular weight substances
 - Hydrogen peroxide (H_2O_2)
- Bacteriocins

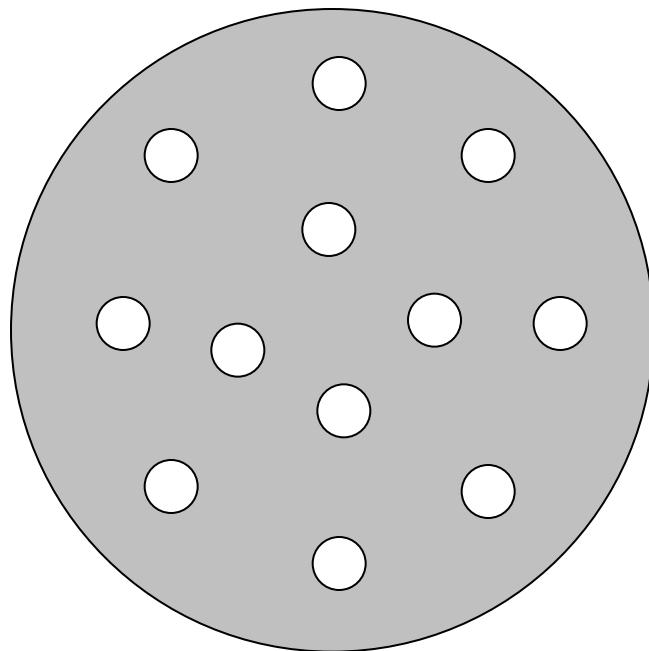


Detection of Inhibition by organic acids in Agar Well Diffusion Assay (ADA)

Supernatant



Soft Agar with indicator strain
Agar



Supernatant without pH adjustment

Supernatant neutralized to pH 7

Smaller inhibition zone size in neutralized supernatant indicate organic acids as inhibitors

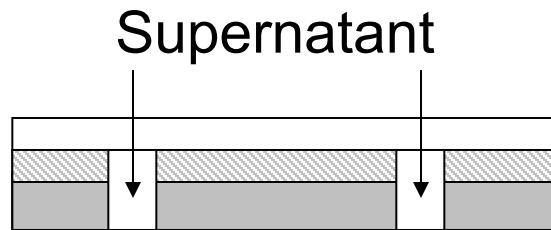


Antimicrobial compounds from LAB

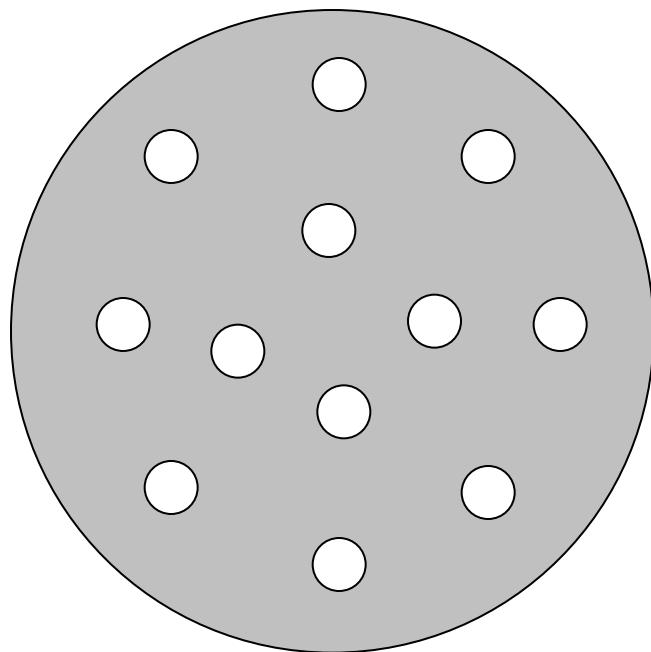
- Organic acids
 - Lactic acid, acetic acid
- Low molecular weight substances
 - Hydrogen peroxide (H_2O_2)
- Bacteriocins



Detection of H₂O₂ Inhibition by Agar Well Diffusion Assay (ADA)



Soft Agar with indicator strain
Agar



Supernatant without catalase

Supernatant with catalase added

Zones smaller in catalase treated samples if H₂O₂ is inhibitory

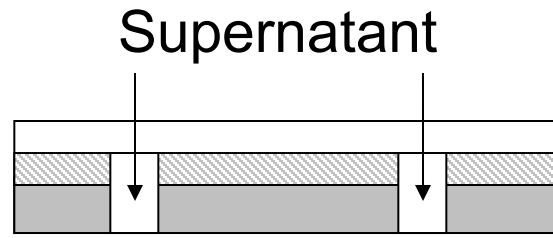


Antimicrobial compounds from LAB

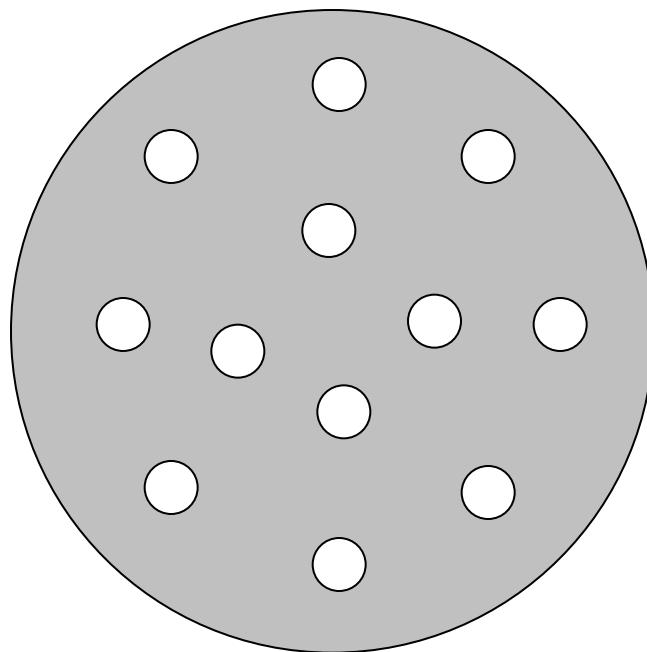
- Organic acids
 - Lactic acid, acetic acid
- Low molecular weight substances
 - Hydrogen peroxide (H_2O_2)
- Bacteriocins



Detection of bacteriocin inhibition by Agar Well Diffusion Assay (ADA)



Soft Agar with indicator strain
Agar



Supernatant without proteinase
(e.g. proteinase K)

Supernatant with proteinase K
added

Zones smaller if proteinase
degrades bacteriocin in
supernatant



Detection and quantification of bacteriocins

Depends on:

- Assay method
 - Detection limit and specificity
- Indicator strain
 - Choice of strain (sensitivity)
 - Media and growth rate
- Producer strain
 - Choice of strain (concentration)
 - Media and growth rate
 - Growth phase
 - Growth temperature



Application



Bacteriocins of LAB in fermentations

Presence

- Direct
 - As pure bacteriocin (e.g. Nisin A)
- Indirect
 - As bacteriocin producing strains or fermentate
- Most LABs are GRAS (Generally regarded as safe)



Applications of LAB bacteriocins (biopreservation) against *Listeria*

- Milk and dairy products
 - Nisin (pure bacteriocin and bacteriocin-producing strains)
 - Outgrowth of clostridial spores (processed cheese)
 - Substitute for nitrate (preservation of cheese, late blowing)
 - Inhibitory to *Listeria monocytogenes* (processed cheese/milk)
 - Pediocin (bacteriocin-producing strain)
 - Inhibitory to *Listeria monocytogenes* (processed cheese)
- Vegetables
 - Nisin (pure bacteriocin and bacteriocin-producing strains)
 - Control of fermentation (Sauerkraut)
 - Sakacin A and Pediocin PA-1
 - Inhibitory to *Listeria monocytogenes* (fermented cabbage)
 - Control of fermentation (Spanish style green olives)
- Meats and meat products
 - Nisin (pure bacteriocin and bacteriocin-producing strains)
 - Inhibitory to *Listeria monocytogenes*
 - Preservation



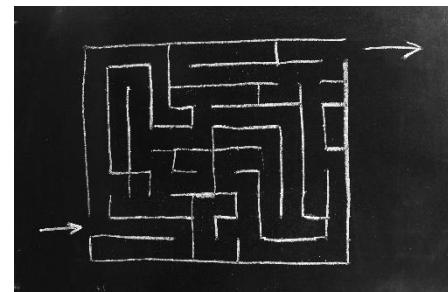
Case Study Title: "The Bacteriocin Battle: A Dairy Dilemma"

Case Background:

In a real-life dairy processing plant, a recurring issue has emerged. Despite following strict hygiene protocols, the plant has been experiencing spoilage problems in their cheese production process. The main culprit appears to be a strain of *Listeria monocytogenes*.

Case Study Scenario:

- Recently, a microbiologist (maybe you) on the plant's team has suggested the use of bacteriocins as an alternative solution to inhibit *Listeria* spp.
- Would you as the team leader implement the use of nisin based on the suggestion? Yes or no? What are the arguments for or against?

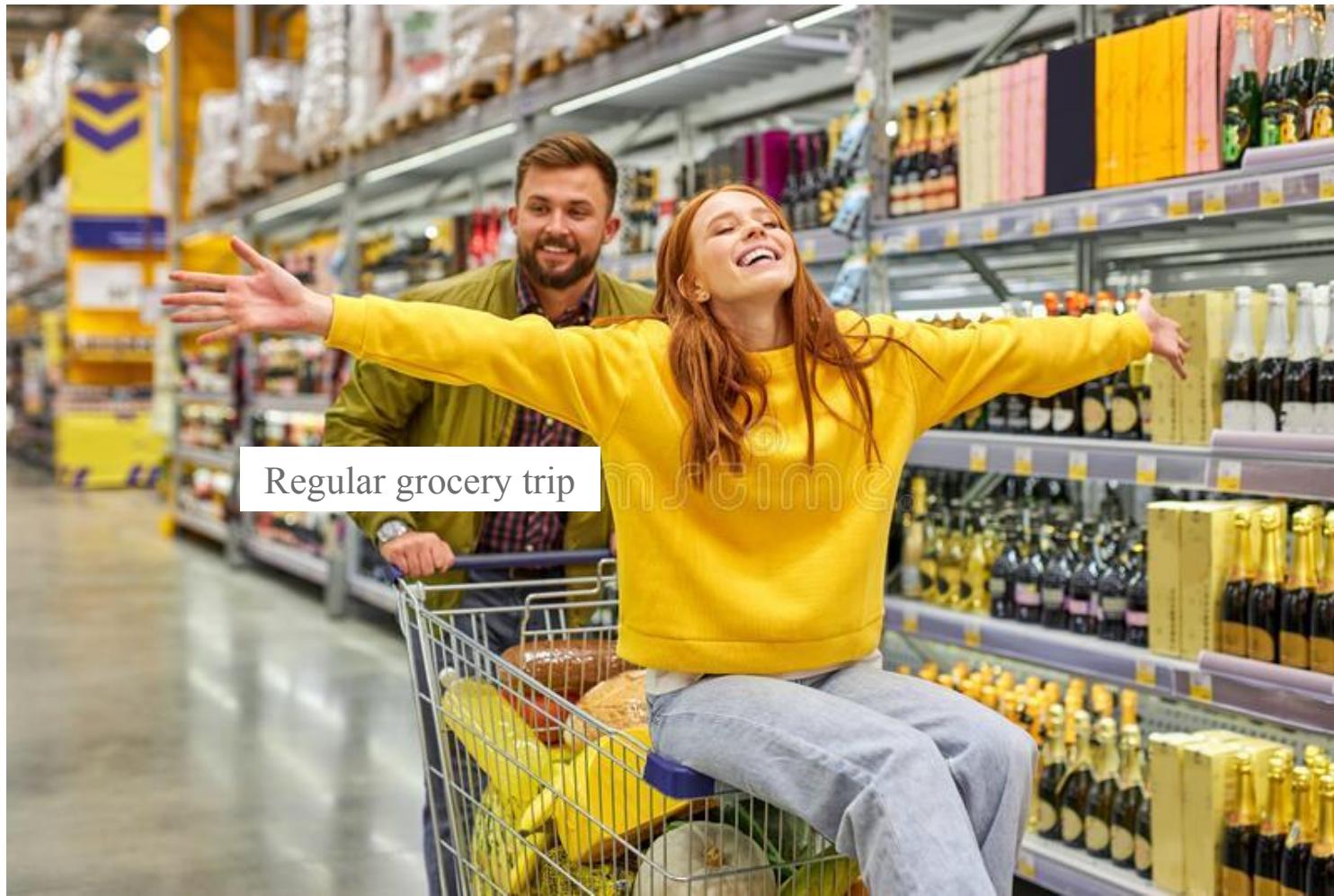


Conclusion - food preservation

- Antimicrobial inhibitor mechanism
LAB: Organic acids, Low molecular weight compounds, and bacteriocins
- Bacteriocins are suitable for food preservation: Qualities are usually pH and heat-tolerant, inactivated by digestive proteases, bactericidal mode, nontoxic to eukaryotic cells
- Biopreservation by microbial origin reduces the amount of chemical preservatives



Bacteriocins – a way to get safe food



Dreamstime.com



Probiotics, prebiotics, synbiotics and postbiotics

Dennis S. Nielsen, dn@food.ku.dk
Department of Food Science

KØBENHAVNS UNIVERSITET



Recap: How to “make” an healthy/unhealthy GM

Diet is the major driver of GM composition/function

Abbreviations:

GPCR = G-protein coupled receptor

PPAR γ = peroxisome

proliferator-activated receptor- γ

PYY = peptide YY

GLP1 = Glucagon-like peptide 1

PAMP = Pathogen-associated microbial pattern

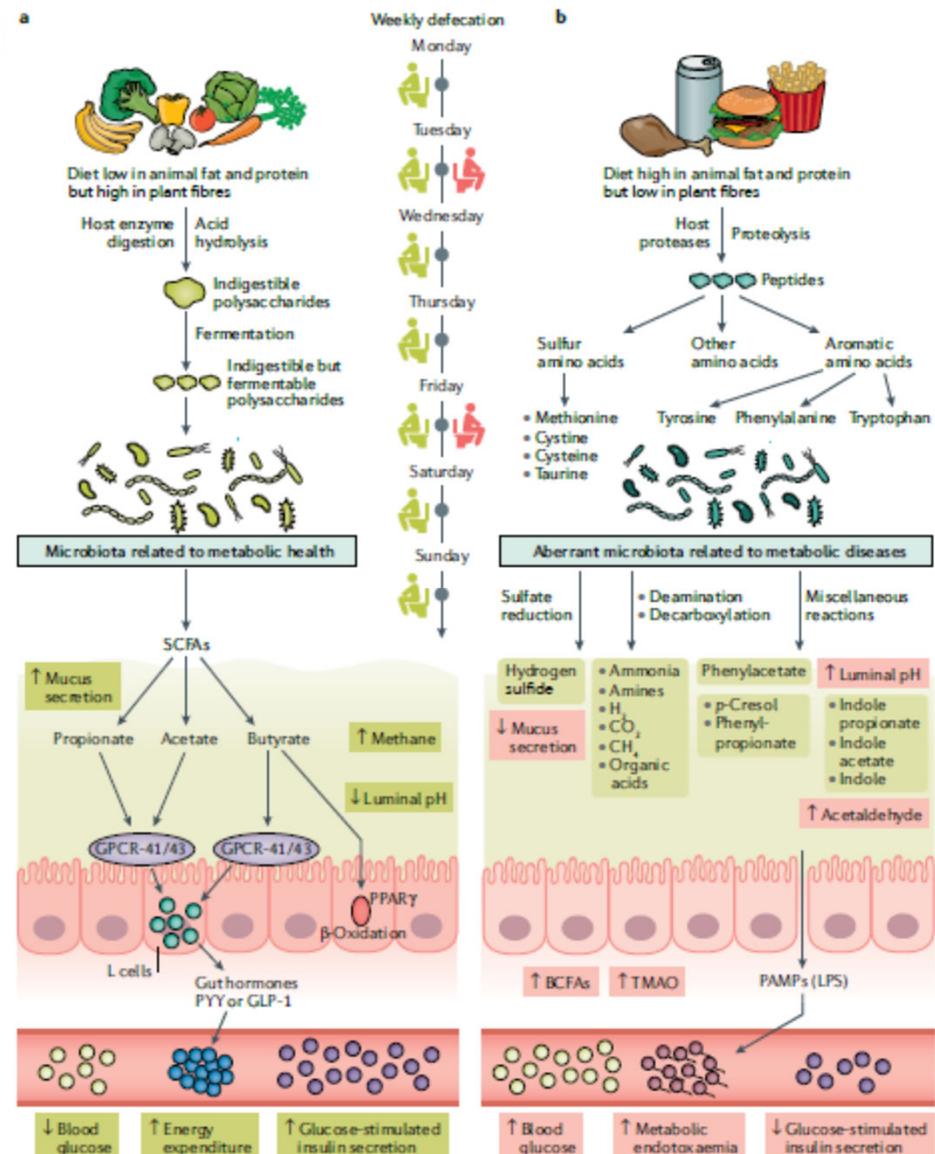
TMAO = trimethylamine N-oxide

Gut microbiota in human metabolic health and disease

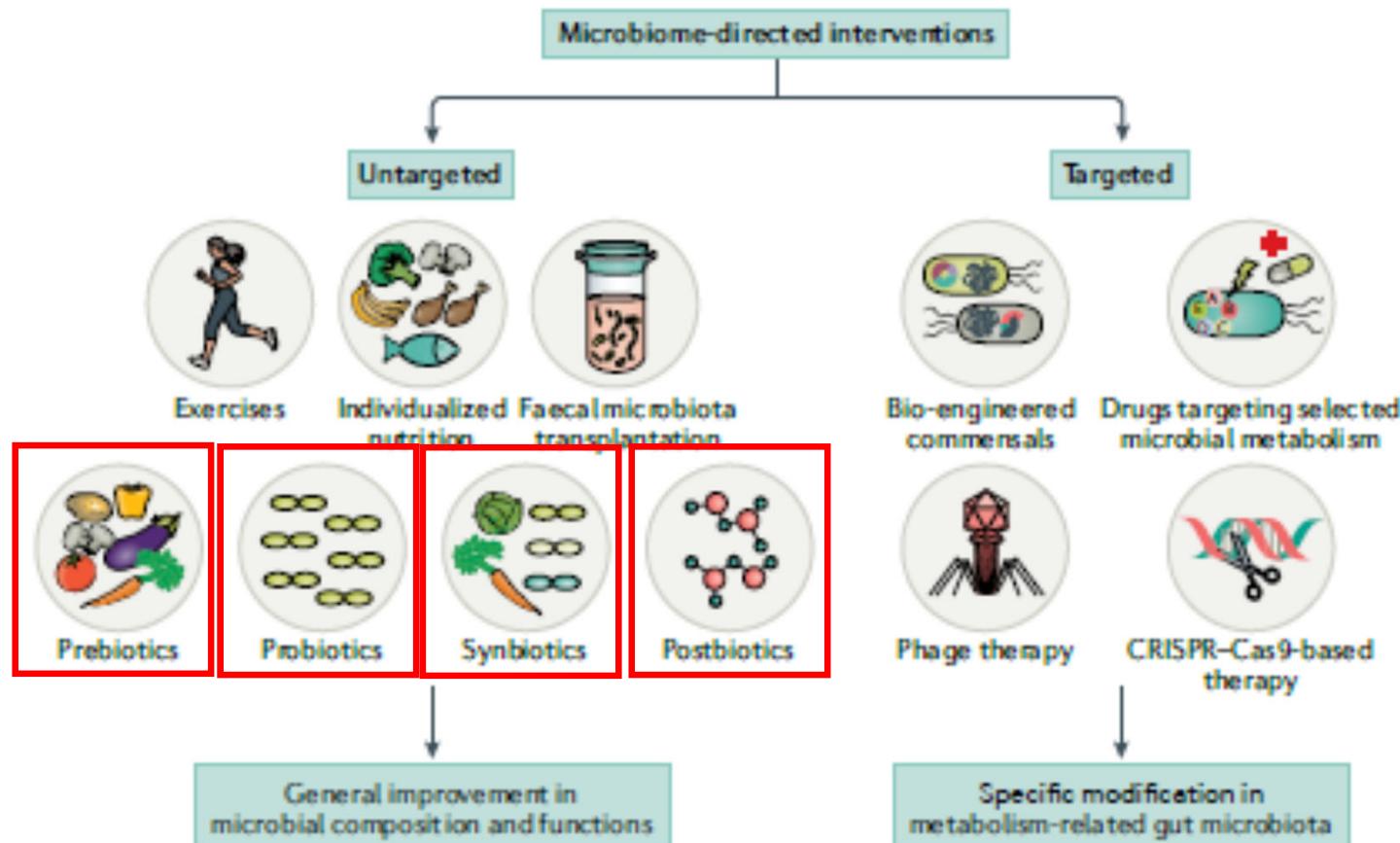
Yong Fan and Oluf Pedersen

NATURE REVIEWS | MICROBIOLOGY

VOLUME 19 | JANUARY 2021



What can you do about it?



Aim of today

- Distinguish **probiotics, prebiotics, symbiotics and postbiotics**.
- Discuss different microbiome-based nutritional concepts to **modulate the gut microbiota composition and activity**
- Discuss the different mode of actions of **probiotics** and **prebiotics** in relation to human health

Probiotics

- Greek: “pro bio” = for life
- The concept of probiotic bacteria normally ascribed to Metchnikoff (around 1905)
- Definition (FAO/WHO 2001)
 - Live microorganisms which when administered in adequate amounts confer a health benefit on the host
- Slightly rephrased in 2014 (but same meaning):
 - Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host

EXPERT CONSENSUS DOCUMENT

The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic

Colin Hill, Francisco Guarner, Gregor Reid, Glenn R. Gibson, Daniel J. Merenstein, Bruno Pot,
Lorenzo Morelli, Roberto Berni Canani, Harry J. Flint, Seppo Salminen, Philip C. Calder
and Mary Ellen Sanders

Nat. Rev. Gastroenterol. Hepatol. **11**, 506–514 (2014)|

Are live microbes the same as probiotics?

- Many probiotics are lactic acid bacteria
- Often LAB are used to make fermented foods
 - Cheese, yogurt, kefir, fermented milks, vegetables, meats, bread, beer, wine
- In some foods, the microbes stay alive until time of consumption → "sources of live cultures"
- BUT: Not all live cultures are probiotics!
- Probiotics must be evaluated and shown to have health effects



Kombucha (fermented tea), 1-10 million microbes pr. gram



Yoghurt (fermented milk), 10 million microbes pr. gram



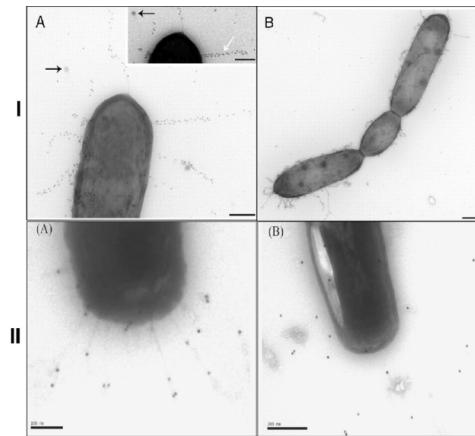
Sauerkraut (fermented white cabbage), 1-10 million microbes pr. gram

Species with (claimed) probiotic members

"Lactobacillus" spp.	<i>Bifidobacterium</i> spp.	Other LAB	'Non-lactics'
<i>L. acidophilus</i>	<i>B. adolescentis</i>	<i>Ent. faecalis</i>	<i>Bacillus</i> spp.
<i>L. amylovorus</i>	<i>B. animalis</i>	<i>Ent. faecium</i>	<i>E. coli</i> Nissle
<i>L. casei</i>	<i>B. bifidum</i>	<i>Ped. acidilactici</i>	<i>Sacch. cerevisiae</i>
<i>L. crispatus</i>	<i>B. breve</i>	<i>Propionibacterium</i>	(= <i>Sacch. boulardii</i>)
<i>L. gallinarum</i>	<i>B. infantis</i>	<i>freudenreichii</i>	
<i>L. gasseri</i>	<i>B. lactis</i>		
<i>L. johnsonii</i>	<i>B. longum</i>		
<i>L. paracasei</i>			
<i>L. plantarum</i>			
<i>L. reuteri</i>			
<i>L. rhamnosus</i>			

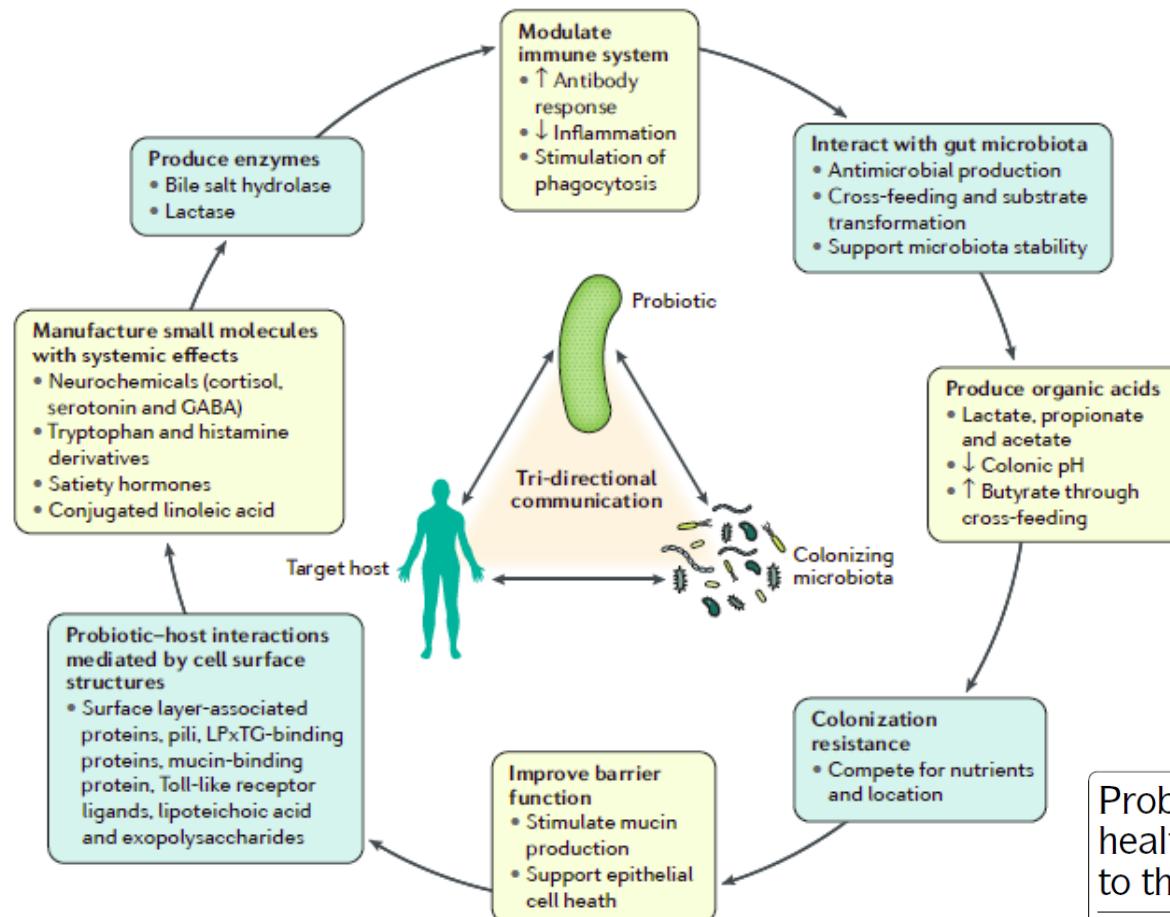


L. acidophilus NCFM



L. rhamnosus GG

Proposed probiotic mechanisms

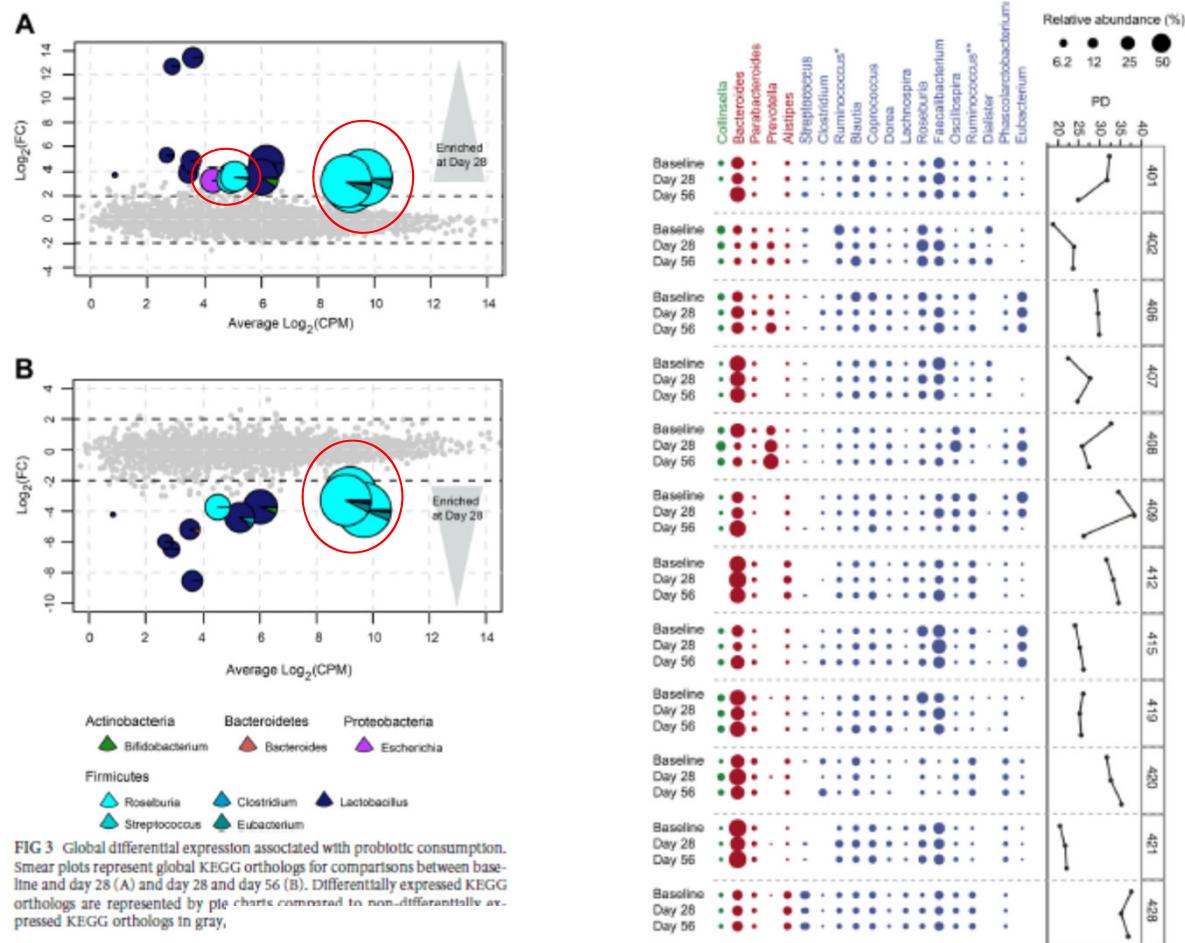


Probiotics and prebiotics in intestinal health and disease: from biology to the clinic

Mary Ellen Sanders¹, Daniel J. Merenstein², Gregor Reid³, Glenn R. Gibson^{4*} and Robert A. Rastall⁴

Probiotics for elderly. Single/multi-strain probiotics

- Some studies indicate that reduced GM diversity is associated with frailty in elderly
- Single strain probiotics do not influence GM to any large extent
- Here *Lactobacillus rhamnosus* GG (LGG) administered to elderly (65-80 years) for 28 days.
- But LGG seems to influence expression profile of GM



Single/multistrain probiotics

- Multi-strain probiotics the way to go to increase diversity?
 - HOWARU Restore (mixture of *Lactobacillus acidophilus* NCFM, *Lb. paracasei* Lpc-37, *Bifidobacterium lactis* Bi-07 and *B. lactis* Bl-04) reduce antibiotic associated diarrhoea (AAD) in hospitalised adults
 - And tend to reduce *Clostridium difficile* AAD (Ouwehand et al., 2014, Vaccine)
- Recently completed study at FOOD: HOWARU Restore to elderly (75 years or older, n = 100)
 - Very limited effect on GM composition
 - Reduced flatulence

Kristensen et al. *Genome Medicine* (2016) 8:52
DOI 10.1186/s13073-016-0300-5

Genome Medicine

RESEARCH

Open Access



Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: a systematic review of randomized controlled trials

Nadja B. Kristensen*, Thomas Bryrup, Kristine H. Allin, Trine Nielsen, Tue H. Hansen and Oluf Pedersen



No or limited effect on GM composition



Article

Randomised, Placebo-Controlled Investigation of the Impact of Probiotic Consumption on Gut Microbiota Diversity and the Faecal Metabolome in Seniors

Gabriella C. van Zanten¹, Anne Lundager Madsen¹ , Christian C. Yde², Lukasz Krych¹ , Nicolas Yeung³ , Markku T. Saarinen³ , Witold Kot⁴, Henrik Max Jensen², Morten A. Rasmussen^{1,5}, Arthur C. Ouwehand¹ and Dennis S. Nielsen¹

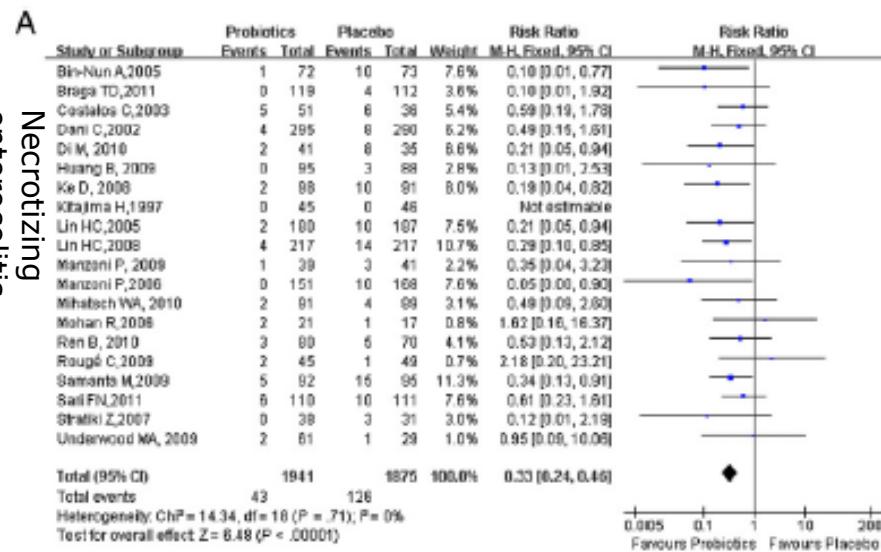


Probiotics for preterm babies

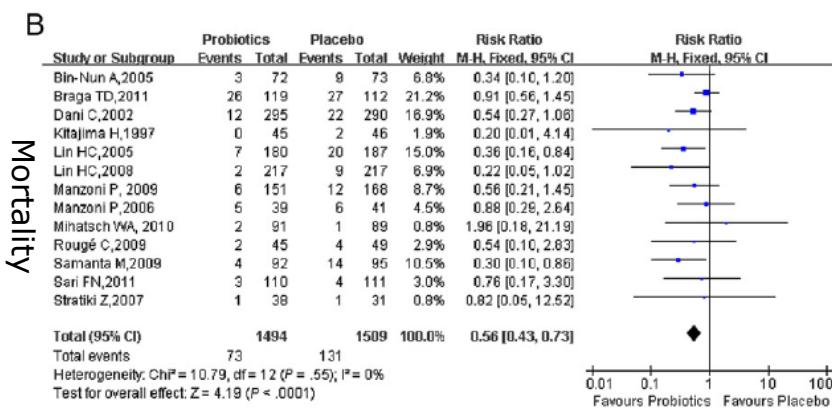
- Infants born prematurely are born with a immature gastrointestinal tract as well
- This is especially the case for infants born very premature (gestational age week 30 or lower)
- Many complications, e.g Necrotizing Enterocolitis (NEC, 5-7% of very low birth weight infants) and sepsis
- GM development of this vulnerable group of infants is also disturbed/altered
 - Probiotics the solution?

Preterms, NEC and probiotics

Necrotizing enterocolitis



Mortality



**Meta-study:
Probiotics
reduce NEC
and
mortality.
No effect on
sepsis**

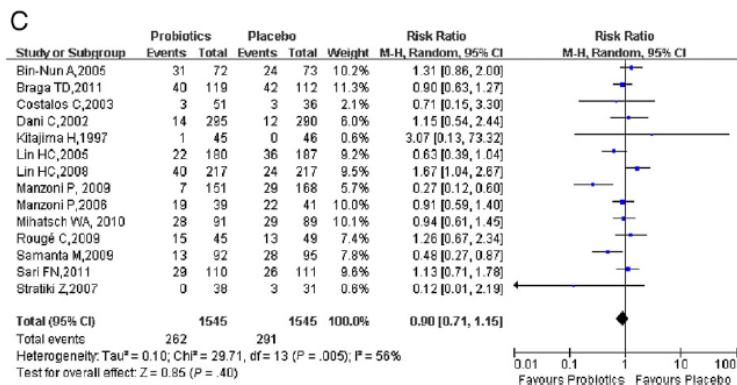


Probiotic supplement reduces risk of necrotizing enterocolitis and mortality in preterm very low-birth-weight infants: an updated meta-analysis of 20 randomized, controlled trials

Quanzen Wang^{a,*}, Jing Dong^b, Yimin Zhu^c

Journal of
Pediatric
Surgery
www.elsevier.com/locate/jpedsurg

Sepsis



Preterm, NEC and probiotics

- But:
- PiPS – Probiotics in Preterm babies Study
- The "study to end all studies" one time for all proving that probiotics are beneficial for preterms
- 1300 preterm babies recruited in double-blinded placebo-controlled randomised clinical trial
- *Bifidobacterium breve* BBG-001
- Result:
 - No adverse effects ☺
 - No effect on NEC, mortality etc. either ☹

***Bifidobacterium breve* BBG-001 in very preterm infants:
a randomised controlled phase 3 trial**

Kate Costeloe, Pollyanna Hardy, Edmund Juszczak, Mark Wilks, Michael R Millar, on behalf of The Probiotics in Preterm Infants Study Collaborative Group*

Lancet 2016; 387: 649-60

PiPS study continued

- But...
 - Low dose ($\approx 5 \times 10^8$ CFU/day)
 - Babies shown to be colonised had better outcome
 - Cross-colonisation to "placebo"-babies (up to 47%...)
 - Wrong (/not the optimal) probiotic strain?
- Remember: No adverse effects, and many studies point to probiotics being beneficial to preterms

	Colonised infants (n=724)	Non-colonised infants (n=462)	Risk ratio (unadjusted, 95% CI)	Risk ratio (unadjusted, 99% CI)	Adjusted risk ratio (99% CI) ¹
Necrotising enterocolitis	47 (7%)	58 (13%)	0.52 (0.36–0.75) p=0.0005	0.52 (0.32–0.84) p=0.0005	0.68 (0.43–1.09)
Sepsis	67 (9%)	66 (14%)	0.65 (0.47–0.89) p=0.0082	0.65 (0.42–0.98) p=0.0082	0.88 (0.59–1.31)
Death before discharge	24 (3%)	33 (7%)	0.46 (0.28–0.77) p=0.0033	0.46 (0.24–0.91) p=0.0033	0.68 (0.35–1.29)

Table: Unadjusted analysis of colonised infants versus non-colonised infants

Deshpande et al., 2016, Lancet

Yeasts as probiotics

- Yeasts differ from LAB and bifidobacteria in many ways
- Yeasts are eukaryotes!
- More diverse enzymatic profiles than LAB and bifidobacteria
- Appear to have a more versatile effect on the immune system (some studies)
- Provide protection against pathogenic bacteria and toxic compounds by surface binding
 - Reduction of diarrhoea quite well established (*Saccharomyces boulardii*)
- Dead cells and particularly fragments of the cell walls are still able to stimulate immune cells

Health claims

- EFSA (European Food Safety Authority) has been very reluctant to approve probiotic health claims
 - Until 2017 no health claims approved in EU
- But in 2014 the Swiss authorities has approved "supports digestive health"-claim (reduces transit time) for *Bifidobacterium lactis* HN019
- First European health claim
- In 2017 EFSA finally approved the first probiotic health claim
 - *Propionibacterium freudenreichii* W200 (vitamin B12)
- The difficulties in getting health claims approved obviously a source of frustration for probiotic producers
- But it is also very difficult to prove that a given probiotic improve the health of healthy persons ("wrong" choice of target populations)
- Be critical: Not everything that calls itself a probiotic has health effects (at least outside Europe...)

Prebiotics

- Prebiotics first defined in 1995
- Definition of a prebiotic:
 - A substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)
- Generally administered orally but could for instance also cover compounds administered to the skin
- Common examples include
 - Fructans (fructooligosaccharides (FOS) and inulin)
 - Galactans (galactooligosaccharides or GOS)
 - But also e.g. resistant starch, some polyphenols etc. live up to the definition

The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics

[doi:10.1038/nrgastro.2017.75](https://doi.org/10.1038/nrgastro.2017.75)
Published online 14 Jun 2017

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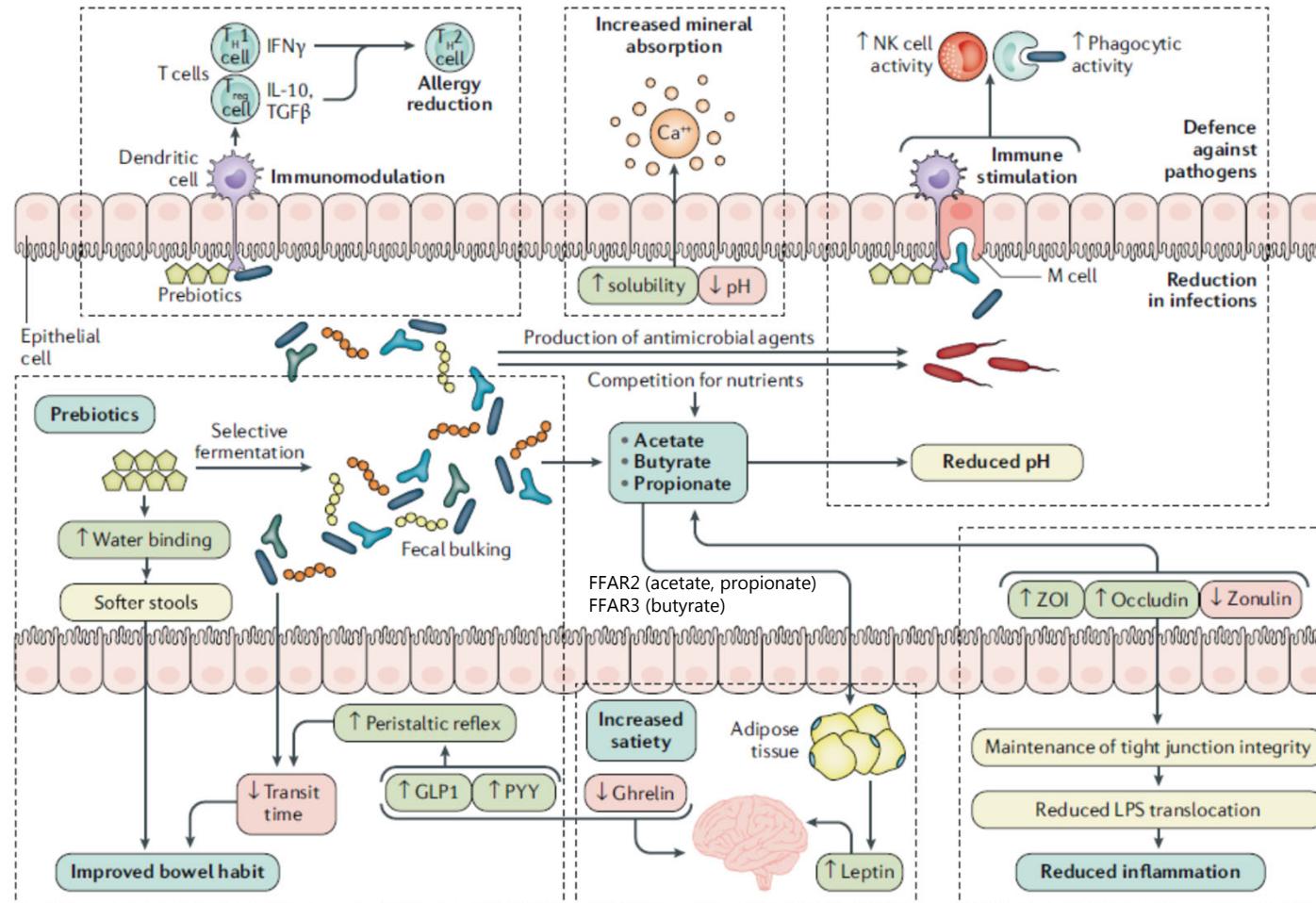
Who to nurture?

Definition: A substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)

Which gut microbes should we selectively feed?

Discuss 2 and 2 for a few minutes. Come up with at least one candidate or desirable property and explain why it is (/could be) a good idea

Prebiotics, proposed mechanism



Abbreviations: GLP1, glucagon like peptide1; M cell, microfold cell; NK cell, natural killer cell; PYY, peptide YY; TGFβ, transforming growth factor-β; TH1 cell, type 1 T helper cell; TH2 cell, type 2 T helper cell; Treg cell, regulatory T cell; ZO1, zonula occludens 1.

A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age

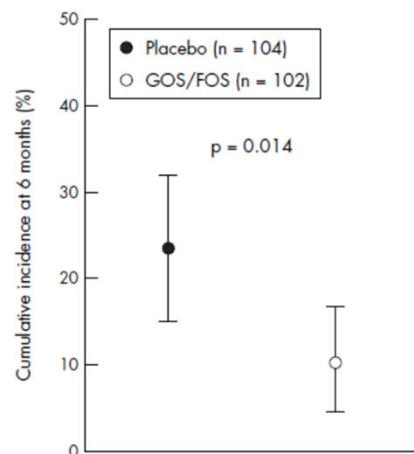
G Moro, S Arslanoglu, B Stahl, J Jelinek, U Wahn, G Boehm



Arch Dis Child 2006;91:814–819. doi: 10.1136/adc.2006.098251

Prebiotics and AD

- Increasing incidence of atopic dermatitis (AD) – prebiotics the solution?
- 259 infants at risk for AD enrolled in RCT. 102 (prebiotic) and 104 (placebo) completed the study
- GOS/FOS mixture, 0.8 g/100 ml infant formula. Maltodextrin as placebo

**Table 4** Stool characteristics at 3 and 6 months of age

	GOS/FOS formula	Placebo formula	p value
n (m/f)	102 (52/50)	104 (49/55)	
At start of formula feeding			
Frequency* (n/day)	2.59±0.9	2.39±1.2	ns
Consistency (score)*	2.3±0.5	2.2±0.5	ns
Infants with score < 2 (n)	20	16	
Infants with score > 4 (n)	0	0	
At 3 months of age			
Frequency* (n/day)	2.34±0.8	1.55±0.6	<0.0001†
Consistency (score)*	2.08±0.5	2.82±0.8	<0.0001‡
Infants with score < 2 (n)	28	0	
Infants with score > 4 (n)	0	0	
At 6 months of age			
Frequency* (n/day)	1.75±0.6	1.50±0.6	0.0059†
Consistency (score)*	2.44±0.7	3.22±0.9	<0.0001‡
Infants with score < 2 (n)	8	0	
Infants with score > 4 (n)	0	9	

*Data are presented as mean ± SD.

†† test.

‡U test.

Table 3 Bifidobacteria and lactobacilli counts as colony forming units (CFU) per gram fresh stool on three consecutive examination days

	GOS/FOS formula	Placebo formula	p value
n (m/f)	50	44	
At start of the study			
Bifidobacteria (CFU/g stool)*	8.17 (2.3)	8.33 (2.4)	ns
Lactobacilli (CFU/g stool)*	4.70 (0.0)	4.70 (0.6)	0.040
At 3 months of age			
Bifidobacteria (CFU/g stool)*	9.56 (0.9)	8.30 (1.1)	<0.0001
Lactobacilli (CFU/g stool)*	6.04 (1.5)	6.12 (2.1)	ns
At 6 months of age			
Bifidobacteria (CFU/g stool)*	10.28 (0.7)	8.65 (1.2)	<0.0001
Lactobacilli (CFU/g stool)*	5.99 (3.6)	5.90 (2.0)	ns

Data are presented as median (interquartile range).

EFSA, prebiotics and health claims

- Contrary to probiotics, the European Food Safety Authorities (EFSA) has approved several health claims for prebiotics such as
 - “Chicory inulin contributes to maintenance of normal defecation by increasing stool frequency”. In order to obtain the claimed effect, 12 g of “native chicory inulin” should be consumed daily”

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Scientific Opinion on the substantiation of a health claim related to "native chicory inulin" and maintenance of normal defecation by increasing stool frequency pursuant to Article 13.5 of Regulation (EC) No 1924/2006

Published: 9 January 2015 Adopted: 11 December 2014



Panel members at the time of adoption

Carlo Agostoni, Roberto Berni Canani, Susan Fairweather-Tait, Marina Heinonen, Hannu Korhonen, Sébastien La Vieille, Rosangela Marchelli, Ambroise Martin, Androniki Naska, Monika Neuhäuser-Berthold, Grażyna Nowicka, Yolanda Sanz, Alfonso Siani, Anders Sjödin, Martin Stern, Sean (J.J.) Strain, Inge Tetens, Daniel Tomé, Dominique Turck and Hans Verhagen.

Abstract

Following an application from BENEOP-Orafti S.A., submitted pursuant to Article 13.5 of Regulation (EC) No 1924/2006 via the Competent Authority of Belgium, the Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on the scientific substantiation of a health claim related to “native chicory inulin” and maintenance of normal defecation by increasing stool frequency. The food constituent that is a subject of a claim is “native chicory inulin”. The Panel considers that “native chicory inulin”, a non-fractionated mixture of monosaccharides (< 10%), disaccharides, inulin-type fructans and inulin extracted from chicory, with a mean DP ≥ 9, is sufficiently characterised in relation to the claimed effect. The Panel considers that maintenance of normal defecation by increasing stool frequency (provided that it does not result in diarrhoea) is a beneficial physiological effect. Six studies involving 86 subjects consistently showed that consumption of “native chicory inulin” at an amount of at least 12 g/day increases stool frequency. The Panel also notes the plausible mechanisms by which inulin and inulin-type fructans in “native chicory inulin” could exert the claimed effect. The Panel concludes that a cause and effect relationship has been established between the consumption of “native chicory inulin” and maintenance of normal defecation by increasing stool frequency. The following wording reflects the scientific evidence: “Chicory inulin contributes to maintenance of normal defecation by increasing stool frequency”. In order to obtain the claimed effect, 12 g of “native chicory inulin” should be consumed daily.

© European Food Safety Authority, 2015

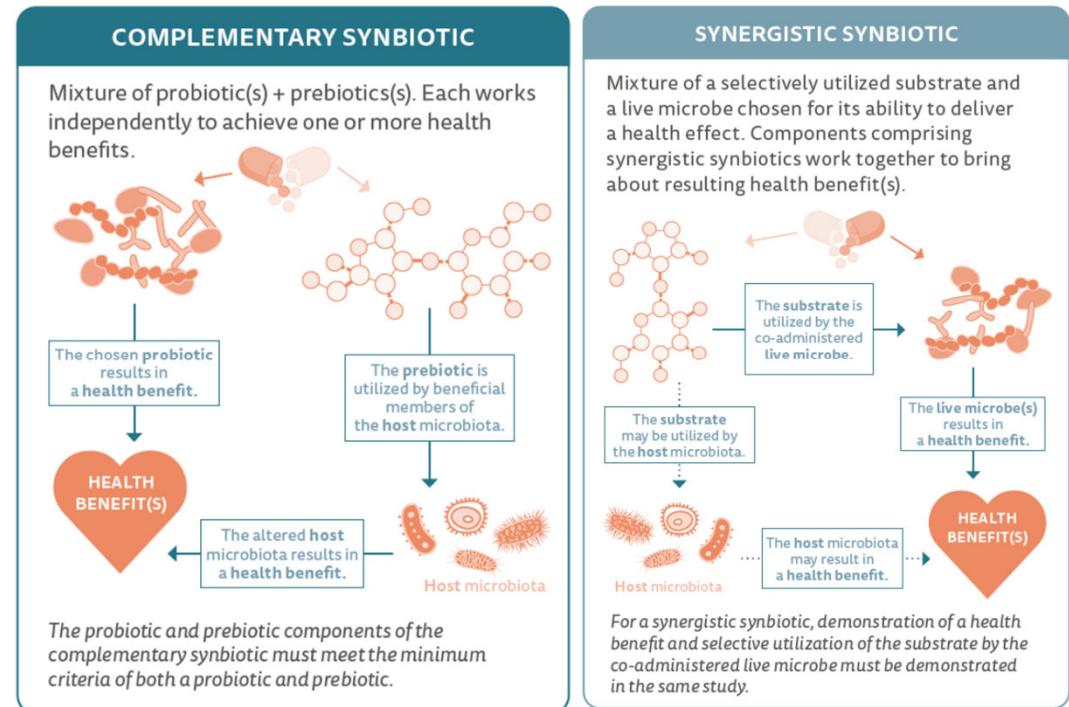
Contents

- Metadata
- Panel members at the time of adoption
- Abstract
- Related topic(s)

Synbiotic

- The concept first conceived in 1995
- Present definition
 - A mixture, comprising live microorganisms and substrate(s) selectively utilized by host microorganisms, that confers a health benefit on the host (Swanson et al., 2020)
- Two types:

Beneficial effect(s) of both complementary and synergistic synbiotics on health must be confirmed in the target host. A study must demonstrate both selective utilization of the substrate and a health benefit.



Synbiotics

Lactobacillus plantarum ATCC 202195 + fructooligosaccharides (FOS)

- 60 days of intervention, 141 rural villages
- Placebo: Maltodextrin

A randomized synbiotic trial to prevent sepsis among infants in rural India

Pinaki Panigrahi^{1,2}, Sailajanandan Parida³, Nimai C. Nanda⁴, Radhanath Satpathy⁵, Lingaraj Pradhan⁶, Dinesh S. Chandel⁷, Lorena Baccaglini⁸, Arjit Mohapatra⁵, Subhranshu S. Mohapatra⁵, Pravas R. Misra⁵, Rama Chaudhry⁸, Hegang H. Chen⁹, Judith A. Johnson¹⁰, J. Glenn Morris Jr¹⁰, Nigel Paneth¹¹ & Ira H. Gewolb¹²

24 AUGUST 2017 | VOL 548 | NATURE | 407

Table 2 | Effect of synbiotic treatment on sepsis and other morbidities in the first 60 days of life

Outcome variables	Control n=2,278 (%)	Synbiotic n= 2,278 (%)	RR (95% CI)	NNT (95% CI)	P value
Death and sepsis (primary outcome)	206 (9.0)	123 (5.4)	0.60 (0.48, 0.74)	27 (19, 47)	<0.001
Deaths	4 (0.2)	6 (0.3)	1.50 (0.42, 5.31)	NA*	0.526†
Sepsis (A + B + C)	202 (8.9)	117 (5.1)	0.58 (0.46, 0.72)	27 (19, 44)	<0.001
A. Sepsis/pSBI—culture-positive septicaemia	27 (1.2)	6 (0.3)	0.22 (0.09, 0.53)	108 (71, 232)	<0.001
Gram-negative sepsis	16 (0.7)	4 (0.2)	0.25 (0.08, 0.75)	190 (110, 699)	0.007
Gram-positive sepsis	11 (0.5)	2 (0.1)	0.18 (0.04, 0.82)	253 (142,1,169)	0.012
B. Sepsis/pSBI— culture-negative sepsis (Culture-negative clinical sepsis warranting hospitalization and IV antibiotics)	36 (1.6)	19 (0.8)	0.53 (0.30, 0.92)	134 (72, 890)	0.021
C. Sepsis/pSBI—LRTI (LRTIs requiring antibiotic therapy)	139 (6.1)	92 (4.0)	0.66 (0.51, 0.88)	48 (30, 126)	0.002
Diarrhoea	59 (2.6)	12 (0.5)	0.20 (0.11, 0.38)	48 (36, 74)	<0.001
Local infections (including >10 pustules, oral thrush, conjunctivitis)	33 (1.5)	16 (0.7)	0.48 (0.27, 0.88)	134 (74, 677)	0.015
Abscess/ otitis media	11 (0.5)	5 (0.2)	0.45 (0.16, 1.33)	NA*	0.133*
Omphalitis	13 (0.6)	3 (0.1)	0.23 (0.07, 0.81)	228 (128,1,045)	0.014

What is
good/bad in
this study?

Synbiotics vs. prebiotics

- Infant formula supplemented with FOS/GOS ± *Lactobacillus paracasei* F19 at 10^6 CFU/ml of infant formula
 - 182 term infants included, followed from day 28 to 6 months of age

Visit 6 (median (IQR))			
	N	65	62
Age (months)		12.10 (12.00, 12.20)	12.10 (12.00, 12.20)
Length (cm)		80.00 (76.95, 83.00)	78.25 (76.50, 82.00)
Weight (kg)		10.24 (9.46, 11.10)	10.26 (9.50, 10.67)
Head circumference (cm)		46.45 (45.50, 47.50)	46.00 (45.25, 47.00)
Weight-for-age z-score		0.96 (0.20, 1.58)	0.85 (0.40, 1.26)
Length-for-age z-score		2.19 (1.36, 3.31)	1.58 (1.03, 2.95)
BMI-for-age z-score		-0.33 (-1.44, 0.46)	-0.00 (-1.76, 0.62)

IQR, interquartile range.

Effects of infant formula supplemented with prebiotics compared with synbiotics on growth up to the age of 12 mo: a randomized controlled trial

Hania Szajewska^a, Marek Ruszczyński^b, Henryk Szymański^b, Iwona Sadowska-Krawczenko^{c,d}, Anna Piwowarczyk^d, Preben Bedstrøp Rasmussen^e, Mette Bach Kristensen^e, Christina E. West^f and Olle Hernell^f

Table 3. Health-related outcomes (intention-to-treat analysis)
—cumulative incidence 0–6 and 0–12 mo

Outcome	Control group (prebiotic formula) (n = 92)	Experimental group (synbiotic formula) (n = 90)	RR (95% CI)
Fever (at least one episode)			
• 0–6 mo	6	8	1.36 (0.5–3.6)
• 0–12 mo	7	10	1.46 (0.6–3.6)
Vomiting			
• 0–6 mo	12	9	0.77 (0.4–1.7)
• 0–12 mo	12	10	0.85 (0.4–1.8)
Eczema			
• 0–6 mo	10	14	1.43 (0.7–3.0)
• 0–12 mo	10	17	1.73 (0.9–3.6)
Gastrointestinal infections			
• 0–6 mo	1	2	2.0 (0.3–15)
• 0–12 mo	3	5	1.7 (0.5–6.3)
Upper respiratory tract infections (at least one episode)			
• 0–6 mo	5	8	1.6 (0.6–4.6)
• 0–12 mo	8	16	2.0 (0.9–4.5)
Lower respiratory tract infections (including wheezing)			
• 0–6 mo	9	5	0.6 (0.2–1.6)
• 0–12 mo	15	5	0.34 (0.13–0.85)
Use of antibiotics			
• 0–6 mo	9	11	1.2 (0.6–2.8)
• 0–12 mo	13	18	1.4 (0.7–2.7)
Unscheduled doctor's visits			
• 0–6 mo	29	30	1.0 (0.7–1.6)
• 0–12 mo	34	40	1.2 (0.8–1.7)
Hospitalization			
• 0–6 mo	6	2	0.3 (0.08–1.4)
• 0–12 mo	10	4	0.4 (0.14–1.2)

CI, confidence interval; RR, relative risk.

Postbiotics

- The newest of the pro-, pre-, syn- and postbiotic concepts. Official definition in 2021:
 - “A preparation of inanimate micro-organisms and/or their components that confers a health benefit on the host”
 - Postbiotics are deliberately inactivated microbial cells with or without metabolites or cell components that contribute to demonstrated health benefits
 - Previous definitions have distinguished between postbiotics (non-viable bacterial products or metabolic products from microorganisms that have biological activity in the host) and parabiotics (non-viable microbial cells (intact/broken) or crude cell extracts which when administered in adequate amounts, confer a benefit on the host – also known as ghostbiotics). Now joined under the above mentioned definition
 - Purified microbial metabolites and vaccines are not postbiotics
 - The site of action for postbiotics is not limited to the gut. Postbiotics must be administered at a host surface, such as the oral cavity, gut, skin…

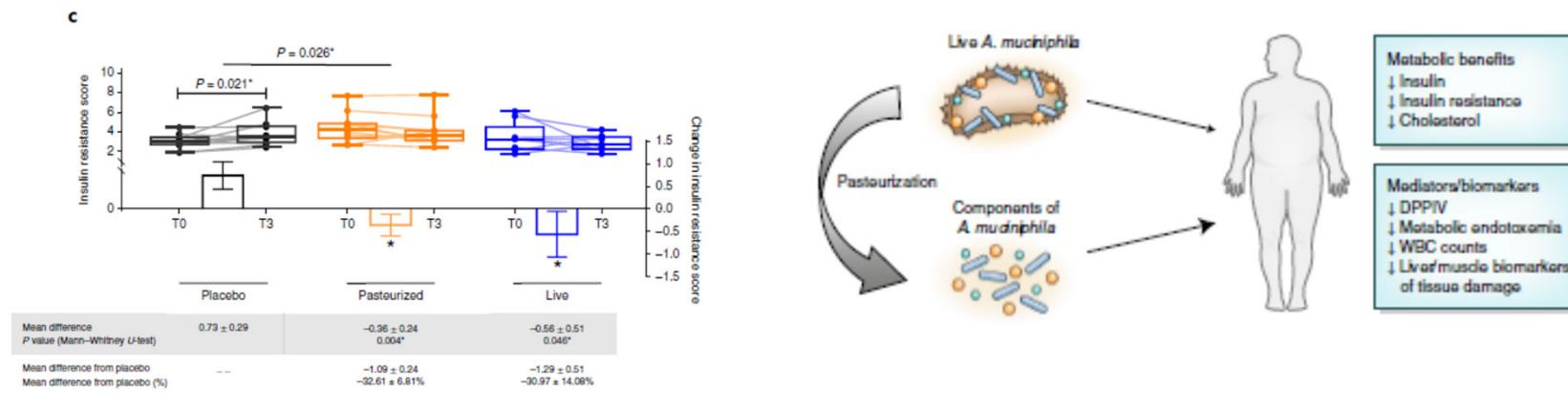
The International Scientific Association
of Probiotics and Prebiotics (ISAPP)
consensus statement on the definition
and scope of postbiotics

Seppo Salminen¹✉, Maria Carmen Collado², Akihito Endo³, Colin Hill^{4,5},
Sarah Lebeer⁶, Eamonn M. M. Quigley⁷, Mary Ellen Sanders⁸, Raanan Shamir^{9,10},
Jonathan R. Swann^{11,12}, Hania Szajewska¹³ and Gabriel Vinderola¹⁴

NATURE REVIEWS | GASTROENTEROLOGY & HEPATOLOGY
SEPTEMBER 2021 | 649

Dead or alive: *Akkermansia muciniphila* to the rescue

- 40 adults with metabolic syndrome (32 completed) randomly allocated to one of 3 treatments for 3 months
 - Placebo
 - 10^{10} live *Akkermansia muciniphila* pr. day
 - 10^{10} pasteurised (dead) *Akkermansia muciniphila* pr. Day
 - Still contain Amuc1100 (a cell membrane protein)



Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study

Clara Depommier^{1,9}, Amandine Everard^{1,9}, Céline Druart¹, Hubert Plovier¹, Matthias Van Hul¹, Sara Vieira-Silva^{2,3}, Gwen Falony^{2,3}, Jeroen Raes^{2,3}, Dominique Maiter^{4,5}, Nathalie M. Delzenne⁶, Marie de Barsy^{4,5,10}, Audrey Loumagne^{4,5,10}, Michel P. Hermans^{4,5,10}, Jean-Paul Thissen^{4,5,10}, Willem M. de Vos^{7,8,10} and Patrice D. Cani^{1,10}*

Conclusion

- Probiotics are safe and might sometimes work
- Prebiotics are a rather well-proven concept for gut micro-biome manipulation with the aim of conferring a health benefit
 - Even products with EFSA approved health claims
- Synbiotics gaining ground
 - Partly because it sounds smart, partly because some studies indicate that there are “additive” beneficial effects
- Postbiotics the new kid in town
 - And then not so new after all – heat treated, fermented foods are kind of postbiotics, too
 - Some benefits (e.g. easier storage)
 - Clinical relevant effect in some studies (but far from all)
 - Heat treated *A. muciniphila*



"I CAN'T AFFORD PROBIOTICS...HOW MUCH FOR AMATEURBIOTICS?"

Bacillus classification, physiology and use in fermented foods

Nadja Larsen, Ph.D.,
Department of Food Science,
Food Microbiology

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1

IJSEM International Journal of Systematic and Evolutionary Microbiology

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Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria (AEB)

N. A. Logan¹, O. Berge², A. H. Bishop³, H.-J. Busse⁴, P. De Vos⁵, D. Fritze⁶, M. Heyndrickx⁷, P. Kämpfer⁸, L. Rabinovitch⁹, M. S. Salminen¹⁰, L. Seldin¹¹ and A. Ventosa¹²

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Abstract

Minimal standards for describing new taxa within the aerobic endospore-forming bacteria are proposed, following Recommendation 30b of the Bacteriological Code (1990 Revision). These minimal standards are recommended as guidelines to assist authors in the preparation of descriptions for novel taxa. They encourage broad polyphasic characterization and the construction of descriptions that are practically useful in routine diagnostic laboratories. The proposals have been endorsed by the Subcommittee on the Taxonomy of the Genus *Bacillus* and Related Organisms of the International Committee on Systematics of Prokaryotes.

A detailed listing of the aerobic, endospore-forming bacteria, together with a brief historical account of the taxonomy of these organisms, and a list of relevant curated 16S rRNA gene sequences of aerobic endospore-formers and relatives are available as supplementary material with the online version of this paper.

2

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Proposed minimal standards for describing new taxa of aerobic,
endospore-forming bacteria

N. A. Lepš¹, O. Bergé², A. H. Böckeler³, H.-J. Böckeler³, P. De Vos², D. Frézey², M. Heyndrickx², P. Kampfer², I. Kämpfert²,
M. S. Sørensen¹, J. Sette² and A. Venema²

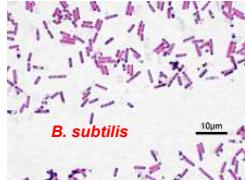
Standard for describing new taxa of AEB

- Isolates:** 5 – 10 strains for each taxon, from several sources/locations
- Microscopic morphology:** Cell size/form, motility/ flagellation, sporangium, position/shape of spore, etc.
- Macroscopic morphology:** Colony description (diameter, shape, colour, surface, hemolysis, etc.) - depend on growth conditions
- Physiological characters:** Growth medium, optimum pH/temp., oxygen requirements, Gram reaction, catalase, oxidase tests
- Biochemical characters:** Acid/gas production from carbohydrates, hydrolysis of casein and starch, citrate utilization, etc.
- Nucleic acid studies:** Sequence analysis (16S rRNA, WGS, protein encoding genes), GC content, DNA fingerprinting
- Reference strains:** Type strains and characterized related strains/ phylogenetic relationship

3

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Taxonomy



Kingdom: Bacteria
 Phylum: Firmicutes
 Class I: Bacilli
 Order I: Bacillales
 Family I: Bacillaceae (47 genera)

Genus: Bacillus ~266 species (highly diverse)

Fermented foods:



Bacillus subtilis
Bacillus licheniformis
Bacillus amyloliquefaciens
Bacillus pumilus

B. megaterium, B. circulans, B. safensis, B. cereus, B. thuringiensis

Medically significant:

B. cereus (foodborne illness)
B. anthracis (causes anthrax)



4

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Physiological characteristics of *Bacillus* spp.

Gram reaction:	Gr +	
Catalase and oxidase:	Positive	
Growth conditions:	<ul style="list-style-type: none"> Aerobic, facultative anaerobic 	
Origin:	<ul style="list-style-type: none"> Fermented foods $\sim 10^{10}$ CFU/g Soil, water, diverse environments Crops and plants (rice, grain, vegetables) 10^2 CFU/g Gut (human) $10^3 - 10^6$ CFU/g feces 	
.		

5

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Cell morphology: Phase contrast microscopy

Bacillus cells/spores:

- Rods (pairs or chains)
- Motile (exception *B. anthracis* and *B. mycoides*)
- Single endospores

B. subtilis group (*B. subtilis*, *B. mojavensis*, *B. clausii*, *B. licheniformis*, *B. sonorensis*)

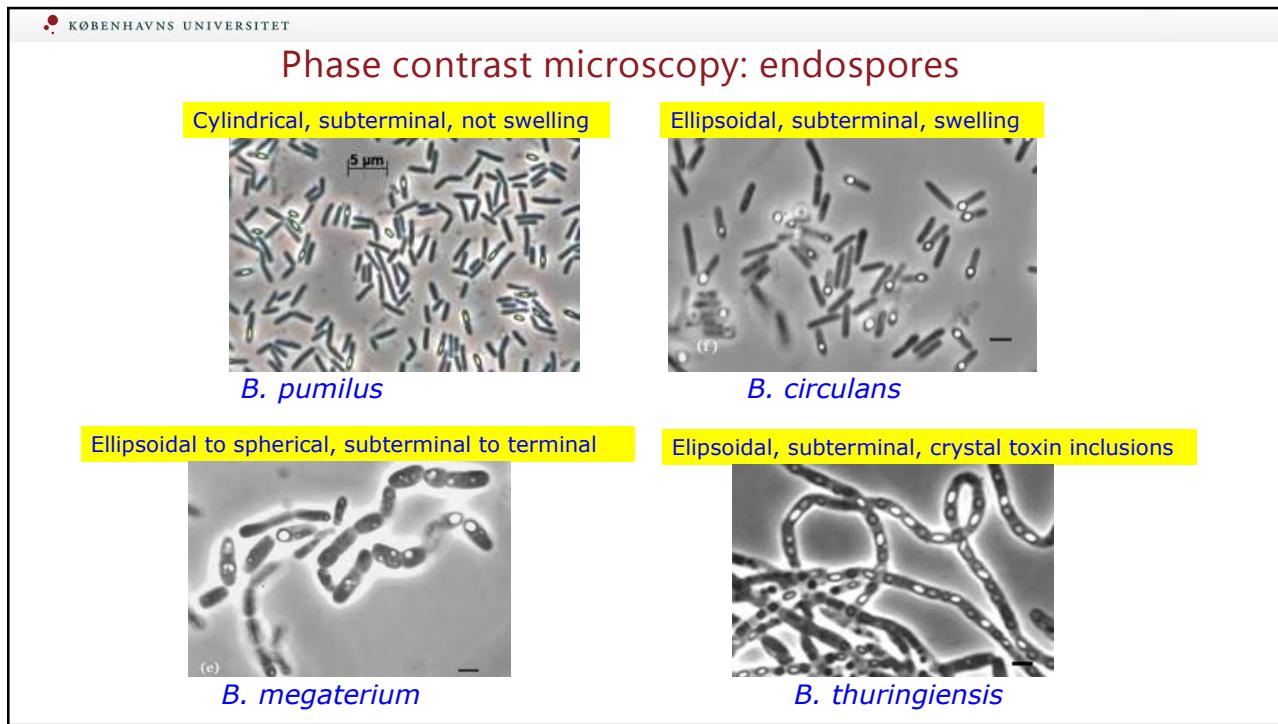
B. cereus

2-4 μm ; sporangia not swollen, ellipsoidal spores

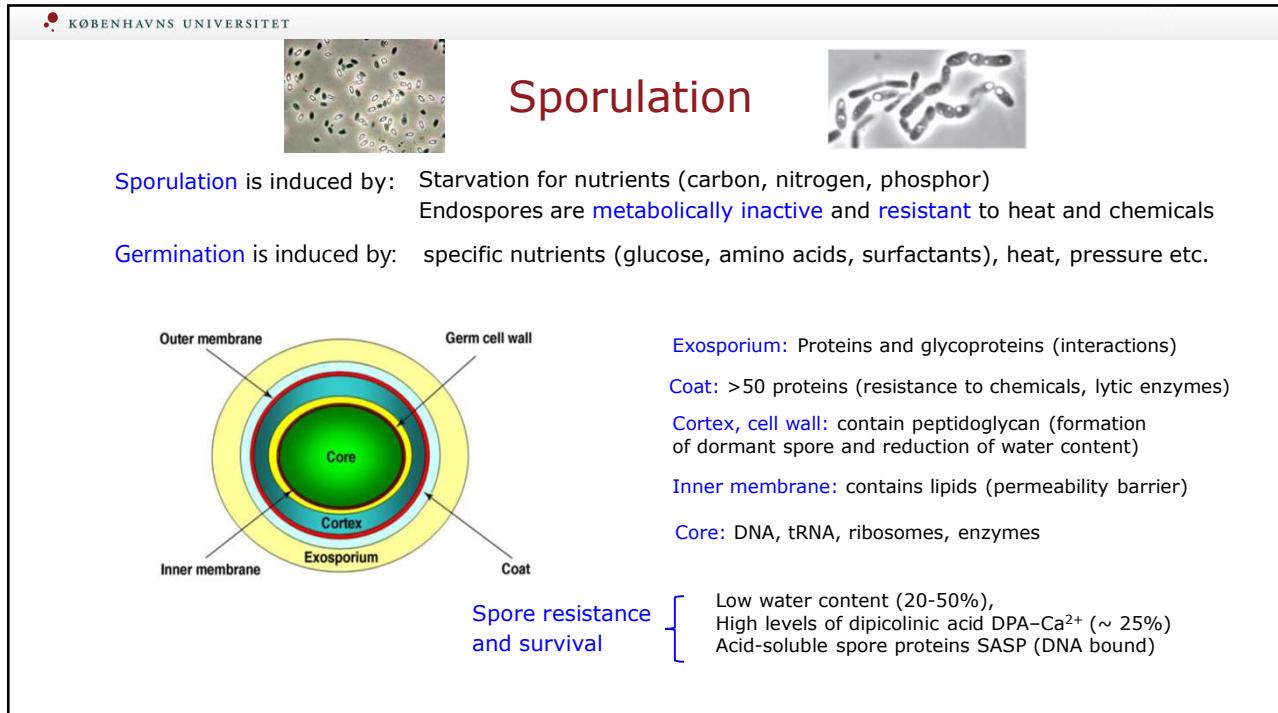
(A) Vegetative cells; (B) aggregated cells with endospores; (C) spores. Bars, 5 μm .

Ynte P. de Vries et al. Appl. Environ. Microbiol. 2004;70:2514-2519

6



7



8

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Hemolysis

- Hemolysis on blood agar
 - Break down of red blood cells (release of hemoglobin)
 - Three types
 - α -hemolysis: reduction of hemoglobin to green methemoglobin
 - β -hemolysis: complete lysis of red cells, clear zones
 - γ -hemolysis: not hemolytic strains



α -hemolysis β -hemolysis

- Toxin production by *B. cereus* group (*B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides*)
 - Hemolysin (HbI)- compound causing hemolysis
 - *B. cereus*: enterotoxin (Nhe) and emeric toxin (cereulide) – associated with food-borne diseases
 - Can't be destroyed by heating



Bacillus subtilis

Other hemolytic bacteria:
Streptococcus ssp.,
Staphylococcus aureus,
Clostridium perfringens

9

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Macro-morphology (Blood Agar)

B. subtilis



B. licheniformis



B. subtilis group:

Large (2-7 mm), opaque, frost-glass appearance, with undulate (wavy) to filamentous margins

B. cereus

Variable morphology
wrinkled and irregular



B. firmus

Small to medium, semi-transparent, flat colonies



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10

Biochemical tests to differentiate between *Bacillus* spp.

- Substrate utilization (glucose, citrate, etc.), enzyme production (hydrolysis of starch, casein, etc.)

e.g. arginin hydrolysis:

B. licheniformis, *B. sonorensis* - positive
B. subtilis - negative

Lecithinase test



B. cereus

Tests for *B. cereus* group (*B. cereus*, *B. anthracis*, *B. thuringiensis*)

- Lecithinase test on egg yolk (lecithin) agar (bacterial phospholipase C cleaves lecithin) - egg yolk precipitation
- MYG (mannitol egg yolk polymyxin) agar test: selective for *B. cereus* (not fermenting mannitol, pink colonies)



MYG agar/manitol:
B. cereus - pink

Antibiotic (penicillin) resistance

- *B. anthracis* sensitive; others *Bacillus* spp. resistant

11

- Related species are difficult to differentiate by phenotypic tests

B. subtilis group (*B. subtilis*, *B. mojavensis*, *B. sonorensis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. vallismortis*, *B. atrophaeus*)

	<i>B. amyloliquefaciens</i>	<i>B. firmus</i>	<i>B. lentus</i>	<i>B. licheniformis</i>	<i>B. pumilus</i>	<i>B. subtilis</i> , <i>B. atrophaeus</i> , <i>B. mojavensis</i> , <i>B. vallismortis</i> , <i>B. velezensis</i>
Motility	+ ^a	v ^a	+	+	+	+
Anaerobic growth	- ^a	-	-	+	-	-
VP reaction	+	-	-	+	+	+
Max. temp. for growth (°C)	50	40–45	35	50–55	45–50	45–50
Egg yolk reaction	-	-	-	-	-	-
Growth at pH 5.7	+	-	-	+	+	+
Hydrolysis of starch	+	+	+	+	-	+
Utilization of citrate	+	-	-	+	+	+
Utilization of propionate	-	-	-	+	-	-
Reduction of NO ₃ to NO ₂	+	+	-	+	-	+

^a+, positive reaction; -, negative reaction; v, variable reaction.

Janet E. L, et al. Handbook of Culture Media for Food and Water Microbiology. 2012

12

➤ After initial/phenotypic characterization use molecular methods (PCR-based)

DNA typing / DNA fingerprinting

by REP-PCR (repetitive extragenic palindromic elements) using e.g. the GTG5 primer
or
by RAPD-PCR (Random Amplified Polymorphic DNA)
using e.g. the PM13-primer (*E.coli* phage-M13 primer)
or
16-23S rRNA internally transcribed spacer PCR (ITS-PCR)

13

➤ Gergoush story

DNA Typing of >160 isolates of *Bacillus* spp. isolated from fermented bread from Sudan (Gergoush)

DNA fingerprinting: M13-PCR /RAPD-PCR

Select representative isolates for 16S rRNA sequencing

Identified species: *B. cereus*, *B. licheniformis*, *B. subtilis*, *B. sonorensis* and *B. thuringiensis*

Thorsen L., 2011. IJFM; 146:244-52.

14

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Taxonomic identification – 16S rRNA sequencing

Why 16S rRNA gene?

- It is ubiquitous, conserved marker
- 16S RNA databases: huge number of sequences for comparison

16S rRNA tree:
Cluster of *B. subtilis* clade

0.002

Bhandari V et al. 2013. J. Systematic Evol. Microbiol.

Drawbacks:

- Closely related species are not necessarily differentiated (16S rRNA gene sequence similarities in *B. subtilis* group 98–99%)
- Multiple 16S rRNA genes in one species/strain (1-2% sequence divergence)

15

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Alternative markers for sequencing *Bacillus* spp.

Select a suitable Core gene/Housekeeping gene:
e.g. *gyrAB*, *rpoB*.... evolve faster than the 16S rRNA gene, but are relatively stable

Separation power of the housekeeping genes can be very good:
e.g. *gyrB* is good for discriminating species in *B. subtilis* group

➤ Drawbacks
Difficult to design suitable primers (because of heterogeneity => degenerated primers for *Bacillus* sp:
gyrB-F 5'-YGGHTATAAAGTTCNGGHGG-3', *gyrB*-R 5'-TCNACRTCBGCRTCBGTCATRAT-3')

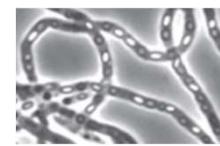
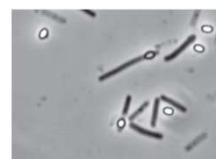
Not so many sequences available for comparisons

16

Summary: Identification of AEB/*Bacillus* spp.

- Macro- and micro-morphology, Gram test, catalase test, sporulation test, other phenotyping/biochemical tests
- DNA typing by molecular based methods
 - Rep-PCR, RAPD-PCR, Species specific "finger-printing"
 - Clustering/grouping (Bionumerics etc.)
- 16S rRNA gene sequencing of representative isolates
 - Identification by BLAST/database search
- Confirm ID and differentiate closely related species by additional tests
 - If possible, sequencing of core genes such as *gyrA*
 - Specific phenotypic tests

17



Bacillus spp. – Use in fermented foods

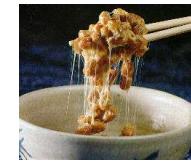


18

Bacillus spp. in alkaline-fermented foods



*Natto, Kinema, doushi,
Thua-nao (from soybeans)*



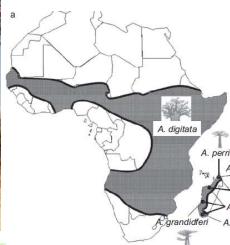
Natto



- Alkaline-fermentations (pH 6-8)
- Spontaneous and controlled fermentations

19

Sources/Raw materials for fermented foods



*Adansonia digitata,
Baobab tree seeds*



*Parkia biglobosa,
African locust bean*

20

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Fermented products and associated microorganisms

Product name/ Country	Raw material	Microorganisms
Dawadawa /Nigeria, Ghana Soumbala /Burkina Faso Iru /Benin, Nigeria	African Locust Bean	<i>B. subtilis, B. pumilus, B.licheniformis, B. thuringiensis, B. cereus, B. megaterium, B. firmis , B. mycoides, etc.</i>
Ugba /Nigeria	African oil bean seeds	<i>B. subtilis, B.coagulans, B. pumilus, B. megaterium</i>
Maari / Burkina Faso, Benin, Mali, Nigeria	Baobab seeds	<i>B. subtilis, LAB: Enterococcus spp., Pediococcus spp.</i>
Natto/Doushi/Thua nao	Soybeans	<i>B. subtilis var natto</i>

Parkouda et al., 2009. Critical Reviews in Microbiology 35, 139-156

21

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AEB occurring in alkaline fermentations

Bacillus subtilis, B. licheniformis, B. amyloliquefaciens

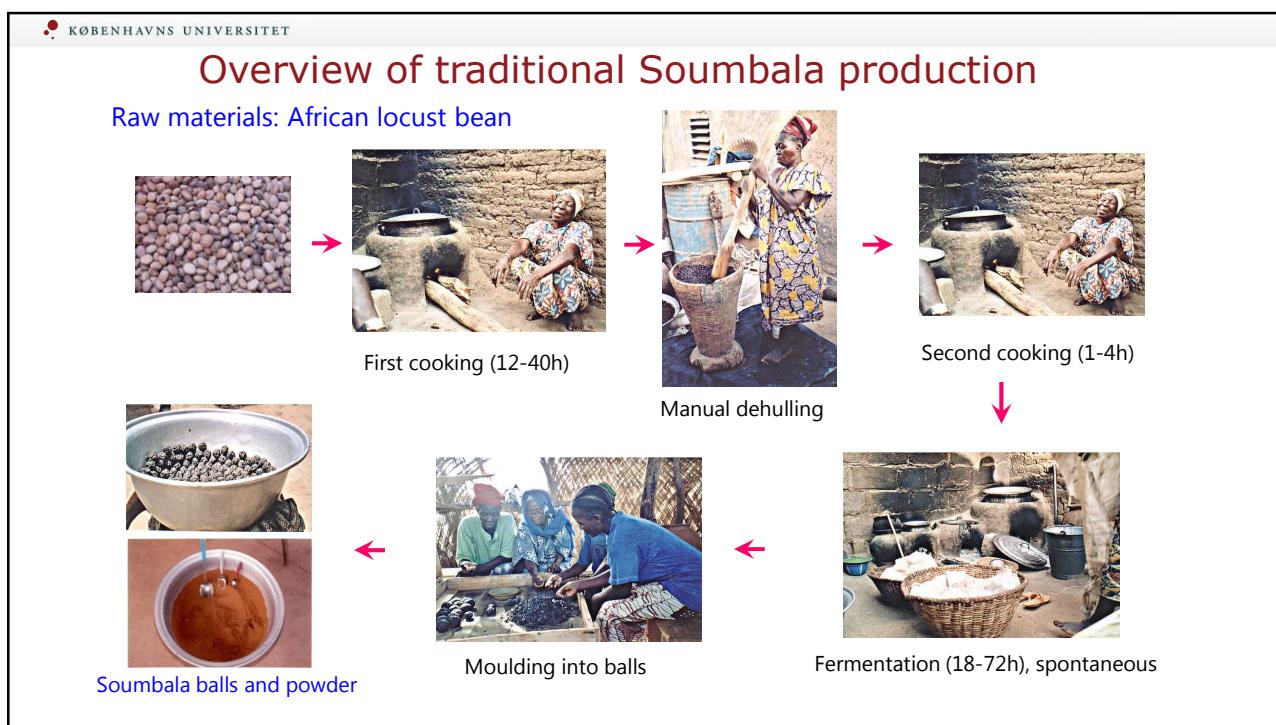
B. pumilus, B. safensis, B. cereus, B. thuringiensis, B. circulans, B. firmus, B. megaterium
.....

Associated microorganisms:

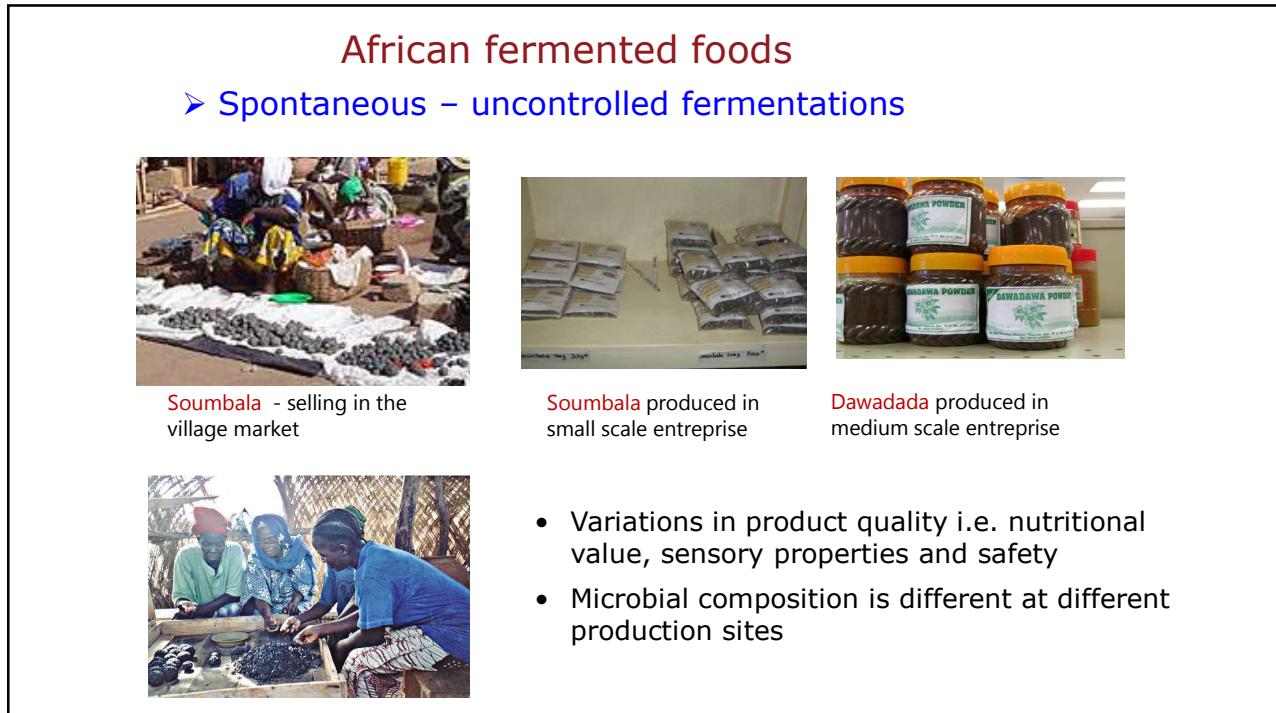
LAB: Leuconostoc mesenteroides, Leuconostoc dextranicus, Pediococcus spp., Enterococcus spp., Micrococcus spp., Pseudomonas aeruginosa, Lysinibacillus sphaericus, Lysinibacillus fusiformis, Brevibacillus spp., Paenibacillus spp., Enterococcus spp., Staphylococcus spp.... And yeasts

Parkouda et al., 2009. Critical Reviews in Microbiology, 35, 139-156.
Parkouda et al., 2010, Int. J. Food Microbiol., 142, 292-301
Darkma et al., 2003. Chana Journal of science, 5, 72-79

22



23

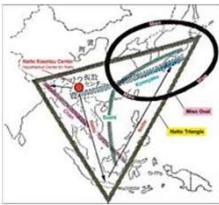


24

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NATTO – an industrialised CONTROLLED fermentation

Triangle of Natto: Japan (Natto), Nepal (Kinema) and Indonesia (Tempeh).



Soybean (*Glycine max*)



Preparation is simple

```

    graph TD
      A([Soybeans]) --> B[Soaked overnight]
      B --> C[Steamed for 30 min]
      C --> D[Inoculated with Bacillus natto]
      D --> E[Incubated at 40°C for 18 hr]
      E --> F[Mature at 5°C for 24 hr]
      F --> G([Natto])
  
```



- Soybeans fermented by *B. subtilis* var. Natto (10^8 CFU/g)
- pH rises from 6.4-6.8 to 7.2-7.6 during fermentation
- The beans become soft and covered by a viscous, sticky polymer, with a distinct musty aroma
- Seasoned and eaten with cooked rice for breakfast

25

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Making inedible foods edible

- Raw materials (seeds, beans)
- ➔ Rich in protein, lipids, sugars, source of amino acids, minerals, vitamins
- ➔ Hard and need to be softened by cooking
- ➔ Not edible directly because of high content non-digestible carbohydrates and anti-nutrients (phytate, oxalate, tannins, trypsin inhibitors etc.)

☞ Fermentation is important for making the raw materials edible, non toxic, improve texture, flavor.. etc.

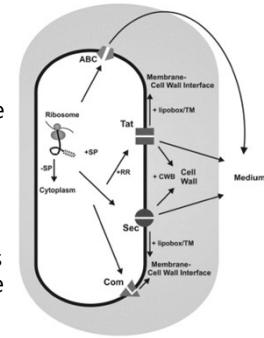



26

Technological properties of *Bacillus* spp. (1)

- Spores are resistant to food processing conditions: e.g. heat
- Production of enzymes
- **Proteolysis:** proteases, peptidases
Increase in peptides and free amino acids
- **Carbohydrate degrading enzymes:** amylases, glucosidases, galactosidases
Degradation of non-digestible carbohydrates, e.g., starch, cellulose and hemicellulose
- **Lipolytic activities:** lipases
Increase in free fatty acids
- **Reduction of antinutrients**
Hydrolysis of phytates (chelate minerals) by phytase, raffinose-family oligosaccharides (cause flatulence) by galactosidases, hydrolyzes cyanogenic glycosides in cassava (tissue damage) by β -glucosidase, degradation of vicin in faba beans (haemolytic anaemia)

=> Increased availability of nutrients



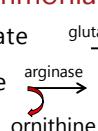
27

Technological properties of *Bacillus* spp. (2)

- Formation of flavour compounds
 - **Volatile flavour compounds (VOC):** aldehydes, ketones, esters, alcohols, alkyl-pyrazines etc.
 - **Non-volatile flavour compounds:** glutamate - umami, and γ -glutamylpeptides - kokumi sensation
- Generation of ammonia (high pH)

$$\text{Glutamate} \xrightarrow{\text{glutamate dehydrogenase}} \alpha\text{-ketoglutarate} + \text{NH}_3$$

$$\text{Arginine} \xrightarrow{\text{arginase}} \text{Urea} \xrightarrow{\text{urease}} 2 \text{NH}_3 + \text{CO}_2$$



- Texture - softening
 - Plant cell wall degrading enzymes (cellulases, xylanases etc.) Hard seeds → Soft fermented seeds
- Texture – viscosity due to production of polymeric substances
 - Levans (β -fructans) – viscous slime material
 - Poly- γ -glutamic acid (PGA) – affects taste, texture, antimicrobial properties



28

Technological properties of *Bacillus* spp. (3)

➤ Biosynthesis of vitamins (vitamin B-group)

- Increased vitamin content (niacin, riboflavinin, thiamin) in fermented soy bean

	Thiamin (B1) mg/kg	Riboflavin (B2) mg/kg	Niacin (B3) mg/kg	Biotin mg/kg
Raw soybeans	16.8	3.4	nd	-
Soaked soybeans	6.9	2.9	nd	-
Cooked soybeans	5.8	6.8	36.4	-
Fermented w. <i>B. subtilis</i> (Kinema)	8.4	11.6	44.8	-
Raw Castor oil Beans	3.2	10.5	5.2	8.9
Cooked unfermented oil beans	1.8	8.4	3.3	7.8
Fermented oil bean (Ogiri)	2.3	26.1	3.1	10.8

Adapted from Sarkar, P.K. et al. 1998. J. Sci. Of Food & Agricul. 78, 498-502

29

Alkaline fermented foods - Summary

- Traditionally produced in Africa and Asia. They are valuable and cheap sources of nutrients (proteins, carbohydrates, essential amino acids, lipids, vitamins) and important source of family income.
- Produced by spontaneous and controlled fermentations. Spontaneous fermentations (Soumbala): Different strains, variations in product quality i.e. nutritional value, sensory properties and safety. Controlled fermentations (Natto): Use of starter culture(s), defined production conditions, expected product obtained each time.
- The technological properties of *Bacillus* spp. include spore heat resistance, enzyme production resulting in release of aroma compounds, degradation of antinutrients, texture improvement, increase of nutrient's bioavailability, vitamins, etc.

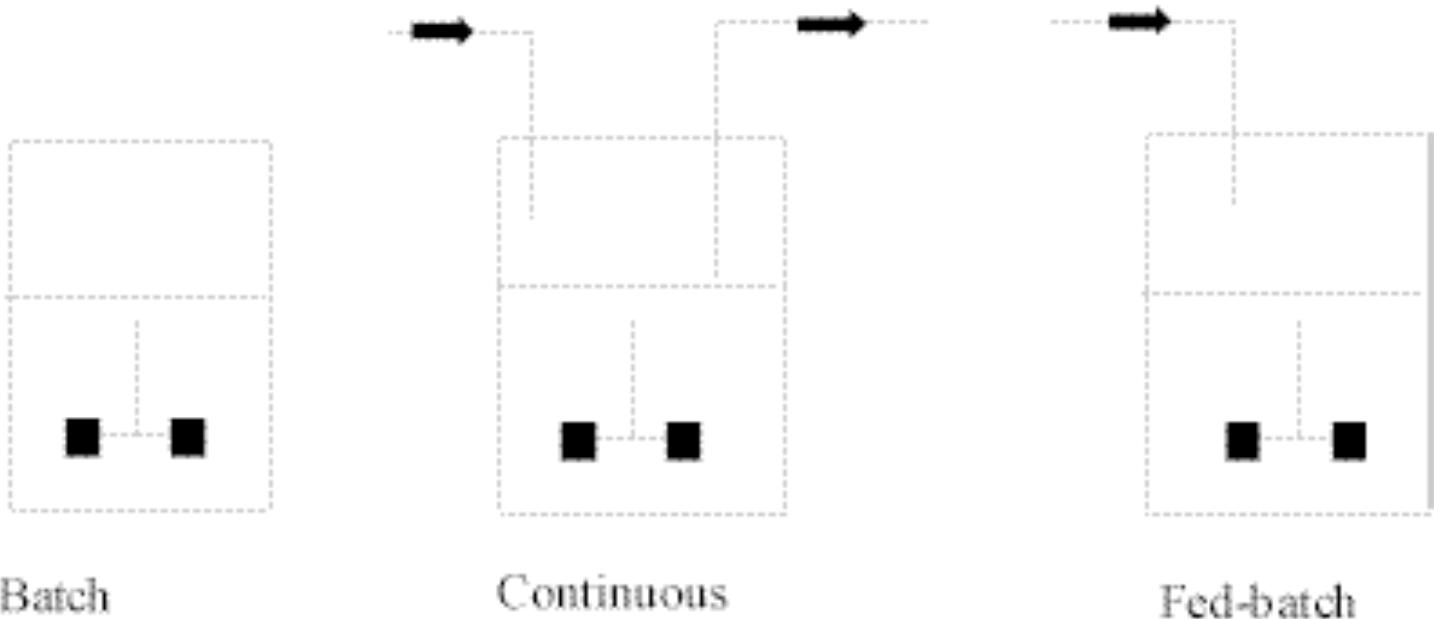
30

Introduction to fermentation processes

Assoc. Prof. Nils Arneborg, FOOD

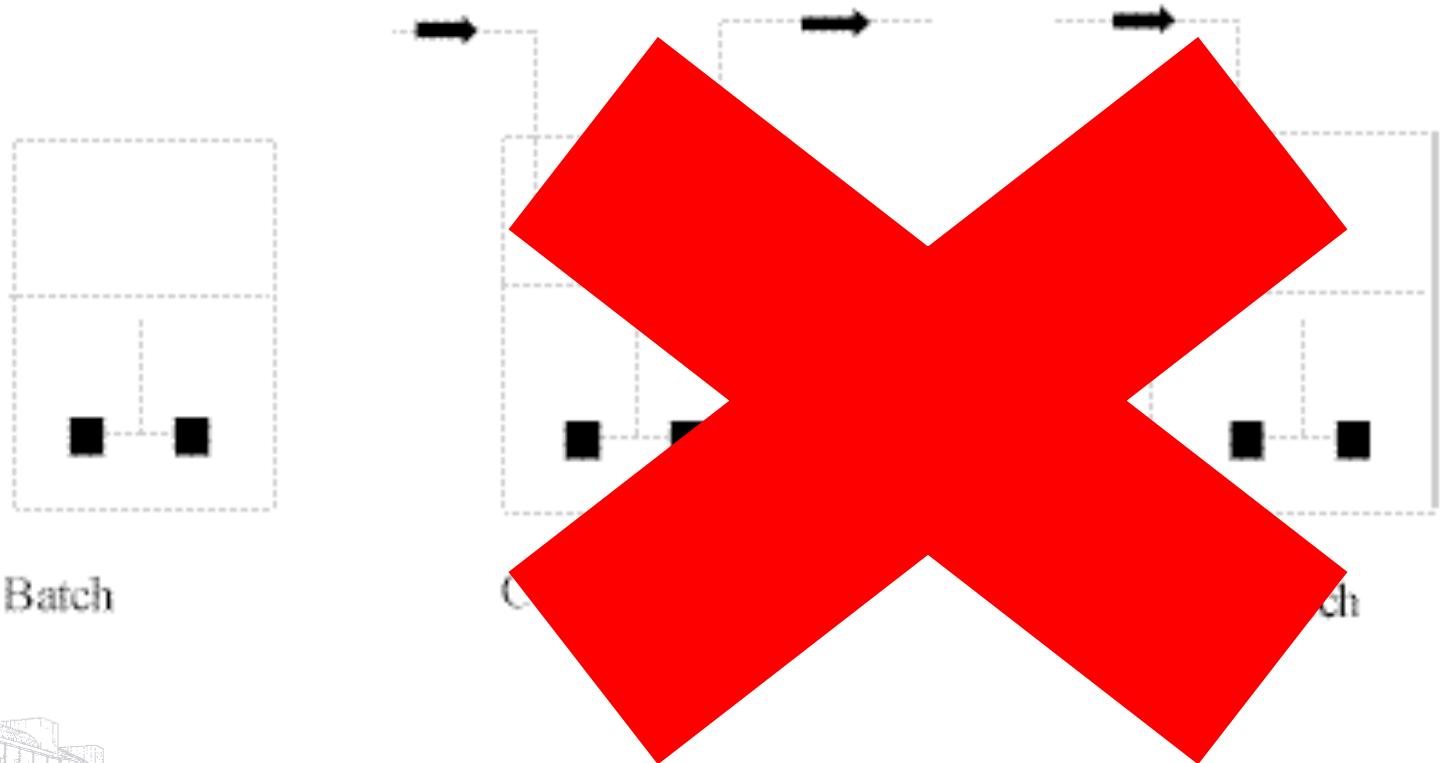


Cultivation techniques



Cultivation techniques

Food fermentations



Fermentation kinetic data

Mass balances

- Specific growth rate

$$\mu = dx/dt \cdot (1/x)$$

[biomass/(biomass•time)]

- Specific consumption rate (of substrate)

$$q_s = ds/dt \cdot (1/x)$$

[substrate/(biomass•time)]

- Specific production rate (of product/metabolite)

$$q_p = dp/dt \cdot (1/x)$$

[product/(biomass•time)]



Fermentation kinetic data

Nomenclature

- x is the **biomass** concentration in the growth medium at time t
- s is the **substrate** concentration in the growth medium at time t
- p is the **product** concentration in the growth medium at time t



Fermentation kinetic data

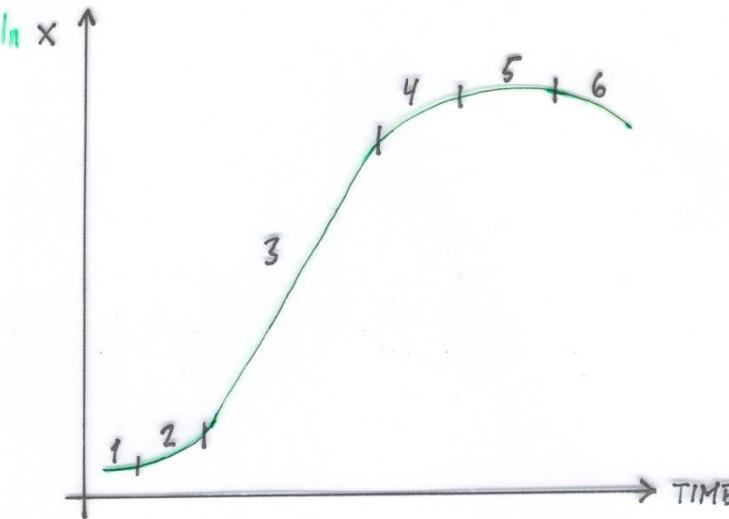
Determination of growth

- Total cell count/microscopic count
- Viable count/plate count/colony count
- Turbidity/optical density
- Cell dry weight



Specific growth rate

Batch



1) Lag phase $\mu = 0$

2) Accelerating phase $\mu: 0 \rightarrow \mu_{\max}$

3) Exponential phase $\mu = \mu_{\max}$

4) Decelerating phase $\mu: \mu_{\max} \rightarrow 0$

5) Stationary phase $\mu = 0$

6) Death phase $\mu < 0$



Fermentation kinetic data

Maximum specific growth rate (batch/exp. phase)

$$\ln x - \ln x_0 = \mu_{max} \cdot t$$

where t is the time

μ_{max} is the maximum specific growth rate

x_0 is the initial biomass concentration in the exponential phase

x is the biomass concentration in the exponential phase



Fermentation kinetic data

*Growth yield factor (**batch**)*

$$\Delta x / \Delta s = Y_{sx}$$

where Y_{sx} is the growth yield factor (e.g. g biomass produced/g substrate consumed)

s is the substrate concentration

x is the biomass concentration



Δ indicates the change of a parameter value within a given time period; e.g. $x_{\text{final}} - x_0$

Fermentation kinetic data

Product yield factor (substrate basis) (batch)

$$\Delta p / \Delta s = Y_{sp}$$

where Y_{sp} is the product yield factor on a substrate basis
(e.g. g product produced/g substrate consumed)

p is the product concentration

s is the substrate concentration

Δ indicates the change of a parameter value within a given time period; e.g. $p_{final} - p_0$



Fermentation kinetic data

*Product yield factor (cell basis) (**batch**)*

$$\Delta p / \Delta x = Y_{xp}$$

where Y_{xp} is the product yield factor on a cell basis (e.g.
g product produced/g biomass produced)
 p is the product concentration
 x is the biomass concentration

Δ indicates the change of a parameter value within a given time period; e.g. $x_{\text{final}} - x_0$



Fermentation kinetic data

*Specific consumption rate (**batch/exp. phase**)*

$$q_s = ds/dt \cdot (1/x) = \mu_{max} \cdot (1/Y_{sx})$$

where q_s is the specific consumption rate during the exponential phase

μ_{max} is the maximum specific growth rate

Y_{sx} is the growth yield factor during the exponential phase



Fermentation kinetic data

*Specific production rate (**batch/exp. phase**)*

Primary metabolite!!

$$q_p = dp/dt \cdot (1/x) = \mu_{max} \cdot Y_{xp}$$

where q_p is the specific production rate during the exponential phase

μ_{max} is the maximum specific growth rate

Y_{xp} is the product yield factor during the exponential phase



Fermentation kinetic data

*Volumetric production rate (**batch/exp. phase**)*

Primary metabolite!!

$$r_p = dp/dt = q_p \cdot x$$

where r_p is the volumetric production rate during the exponential phase
 q_p is the specific production rate during the exponential phase
 p is the product concentration
 x is the biomass concentration
 t is the time



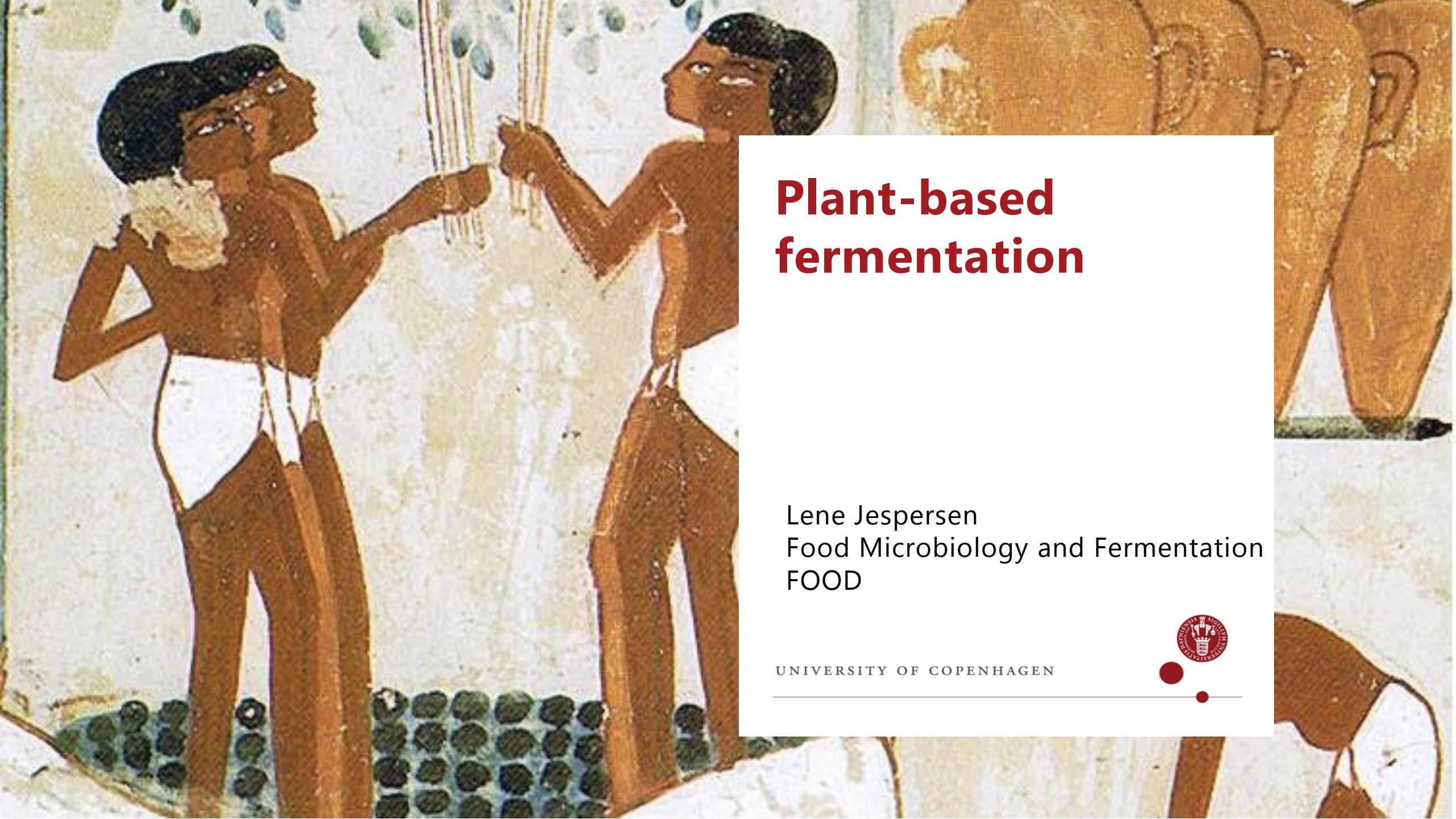
Fermentation kinetic data

*Important tool for assessment of e.g. the
technological properties of microorganisms
used for fermented foods*

Advantages

- Differences are quantified
- Less subjective evaluation
- Statistics may be included
- Correct basis for comparisons



A traditional Egyptian fresco painting depicting two men in a garden. One man, on the left, is shown from the side, holding a bunch of grapes in his right hand and a staff or branch in his left. The other man, on the right, is shown from behind, reaching up towards a vine hanging from above. They are standing on a path with green plants at their feet. The scene is set against a light-colored wall.

Plant-based fermentation

Lene Jespersen
Food Microbiology and Fermentation
FOOD

UNIVERSITY OF COPENHAGEN



Intended learning outcomes

- Knowledge on different types of plant-based fermentations and their characteristics
- Being able to identify and understand the functionality of different microorganisms in plant-based fermentations
- Reflect on the applicability of fermentation for developing new plant-based products

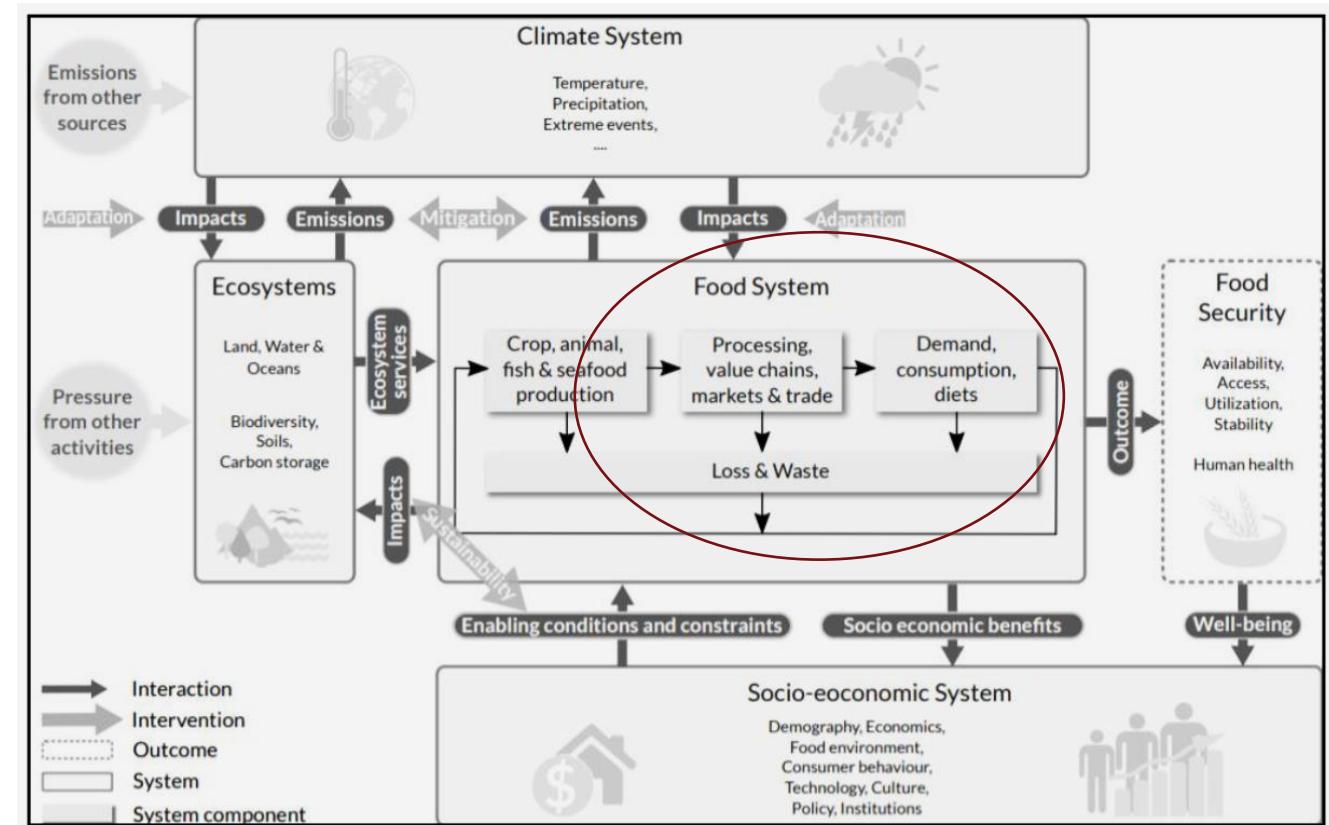
The interrelationship between climate change, food systems and food security

"Food security will be increasingly affected by projected future climate change"
(high confidence, the Intergovernmental Panel on Climate Change, IPCC)

The big ???

How do food borne
microorganisms impact
climate changes and relevant
food systems?

Can microorganisms optimise
plant-based foods?



Current trends in food fermentation

Transition to more (fermented) plant based foods

- Product diversity – new raw materials/new microbes
- Optimisation of taste (umami/kokumi...) and structure (water binding/uptake of nutrients)
- Clean labelling - fermentation as a green technology!
- Solid stage fermentations – a challenge?
- From probiotics to fermented foods
- Prevention of food waste
- Valorisation of side-streams/co-products
- Back to basic – “make you own kombucha”
- Green cities and optimised food systems



2 minutes of own reflection on how fermentation can
be used to enhance the quality of new innovative
fermented plant-based products



Fermentation is a step towards a more sustainable food production

Fermentation can ...

- limit processing steps as e.g. cooking
- prevent loss of raw materials
- enhance food shelf life due to production of antimicrobials, organic acids, bacteriocins etc. and storage of food out of the cold chain
- limit the use of food preservatives by bio-preservation
- prevent food waste due to improved food safety
- make inedible raw materials edible

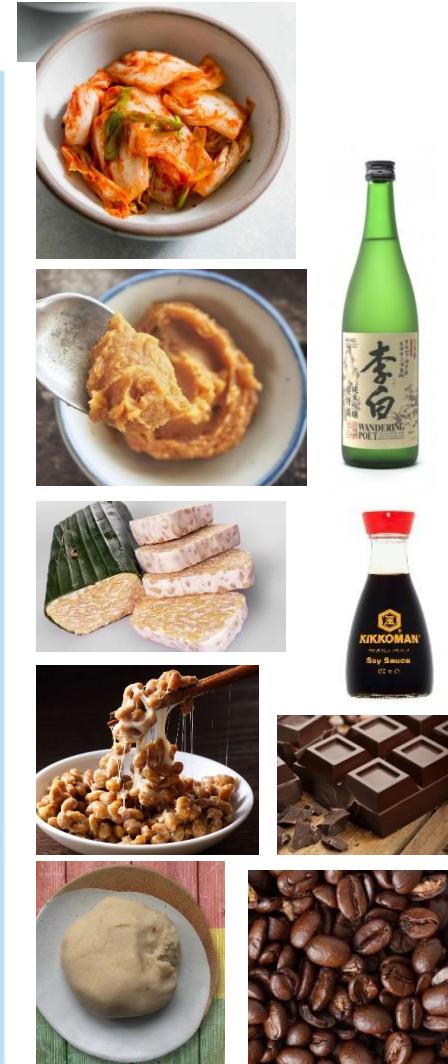
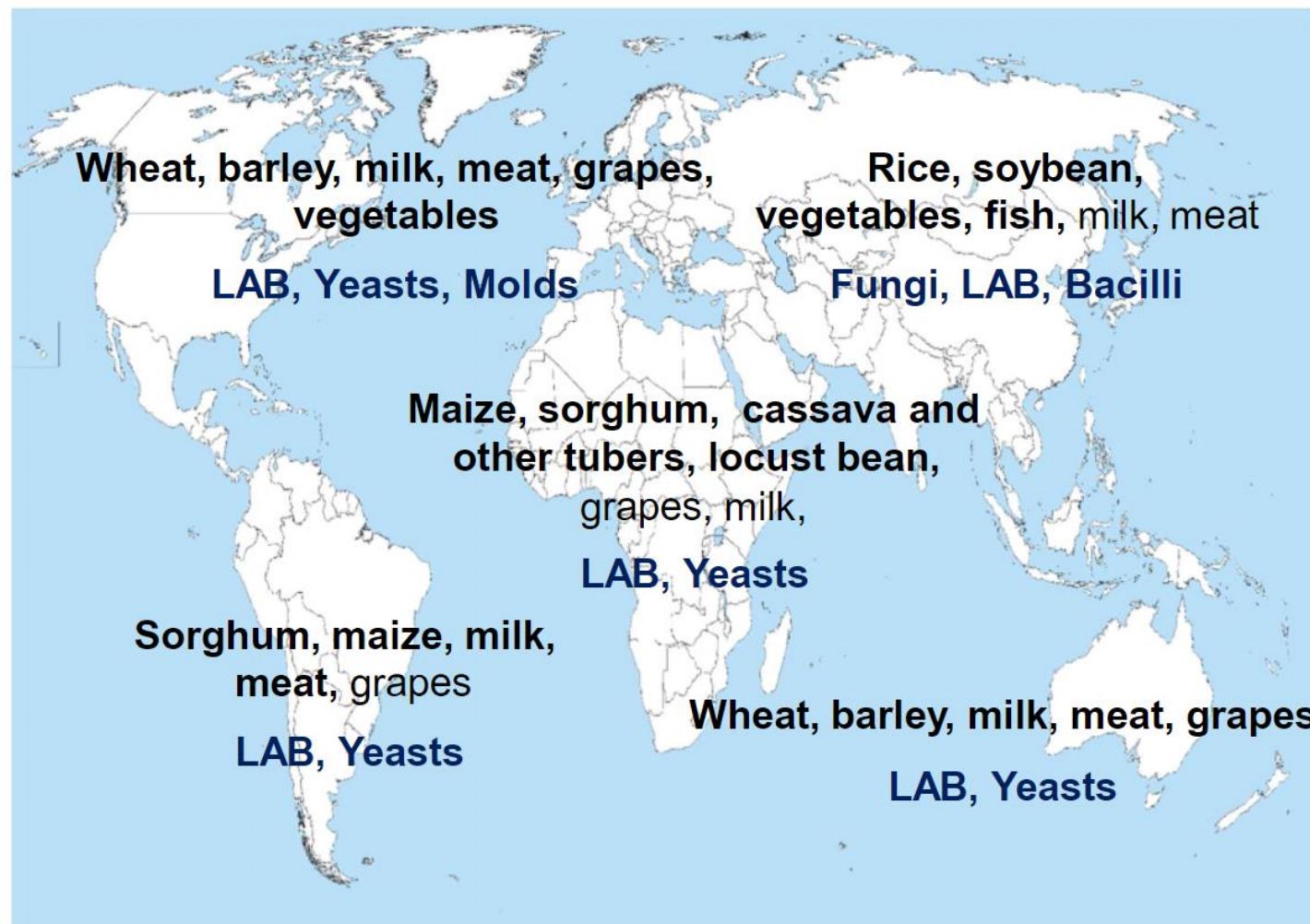


SUSTAINABLE
DEVELOPMENT
GOALS

Food fermentation is a sustainable and green technology !!!

Estimate: 1/3 of the diet worldwide consists of fermented products, plant-based make up the most

- Cereals
- Vegetables
- Legumes
- Roots and tubers
- Fruits



Tempeh – an inspiration for many new Nordic products



Raw materials:

- Soy beans but other beans or cereals have been used ("Nordic" Tempeh could be from rape, barley, pea.....)
- Tempeh mold *Rhizopus oligosporus/Rhizopus oryzae* and lactic acid bacteria

Tempeh is normally consumed fried, boiled, steamed or roasted.

During fermentation → increased amount of:

- free amino acids
- Vitamins (e.g. B12)
- water-soluble nitrogen compounds,
- free fatty acids
- development of characteristic flavour

Decrease in the amount of crude lipids (serve as energy for the microorganisms). The protein hydrolysis may amount to 25% of the initial soy protein (Sparrings & Owens, 1999).

Fermentation may help digestibility (less flatulence), less bitterness from glycosinolates or polyphenols, aid mineral uptake

Production Process of Tempeh

- 1.Cracking the soybeans.
- 2.Soaking and dehulling the soybeans.
- 3.Cooking the soybeans.
- 4.Inoculating the soybeans with tempeh starter.
- 5.Incubating the beans (fermentation process)

Plant based beverages, examples

- Beer, mead, wine, cider: ethanol, acidification, aroma, yeast (and lactic acid bacteria)
- Kombucha: acidification, aroma, (ethanol), Symbiotic culture of bacteria and yeasts (SCOBY)
- Kvass: (a fermented cereal-based low-alcohol beverage with a slightly cloudy appearance) acidification, aroma, (ethanol), yeast and lactic acid bacteria
- Fermented juices: acidification, aroma
- Cash crops:
- Tea: such as pu-erh dark tea, *Aspergillus* mould
- Coffee: pectinolytic activity, aroma, primarily yeast
- Cocoa: removal of bitterness, aroma, sequential bacteria and yeast



Fermentation may expand our food range

Cassava (starchy >70% cal. in Nigeria)



Without
fermentation



- Short shelf life
- High content of cyanogenic glycosides

Fermentation
(LAB, *Bacillus* and
yeast)



- Longer shelf life
- Reduction of cyanogenic glycosides
- Improved flavour



The microbiota of Lafun, an African traditional cassava food product

Sègla Wilfrid Padonou ^{a,b,*}, Dennis S. Nielsen ^c, Joseph D. Hounhouigan ^a, Line Thorsen ^c,
Mathurin C. Nago ^a, Mogens Jakobsen ^c

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ORIGINAL ARTICLE

Development of starter culture for improved processing of Lafun, an African fermented cassava food product

S.W. Padonou¹, D.S. Nielsen², N.H. Akissoe¹, J.D. Hounhouigan¹, M.C. Nago¹ and M. Jakobsen²

Fermentation expands “edibility” e.g. Soumbala/dawadawa/iru

- African locust bean seeds are inedible when raw ☺ what to do?
- Alkaline fermentation (*Bacillus* spp.) turns them into a condiment which:
 - contributes significantly to the intake of: protein, essential fatty acids, and group-B vitamins
 - has flavoring attributes, works like maggi and add umami
 - is an important source of family income
- Fermentation increased sharply total free amino acids and essential free amino acids between 24 and 48h (cysteine, methionine, leucine, isoleucine, tyrosine and phenylalanine).

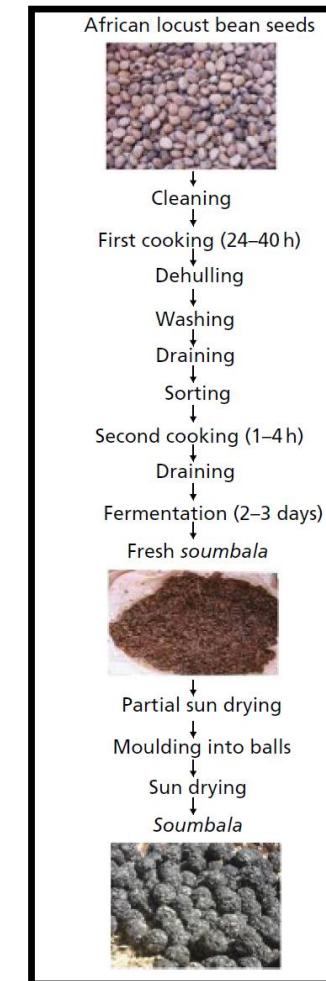
Journal of Applied Microbiology 2003, **94**, 396–402

Degradation of proteins during the fermentation of African locust bean (*Parkia biglobosa*) by strains of *Bacillus subtilis* and *Bacillus pumilus* for production of Soumbala

L.I.I. Ouoba^{1,2}, K.B. Rechinger^{2,3}, V. Barkholt⁴, B. Diawara¹, A.S. Traore⁵ and M. Jakobsen^{2*}

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22 October 2002 and accepted 5 November 2002



Bacillus spp. in spontaneously fermented seed condiments in West-Africa

4 J. OWUSU-KWARTENG ET AL.

Table 1. Overview of predominant *Bacillus* species occurring in fermented seed condiments in West Africa.

Product name	Substrate/raw material	<i>Bacillus</i> spp.	Method of identification	Country/region	Reference
Dawadawa	<i>Parkia biglobosa</i> seed	<i>B. subtilis</i> , <i>B. pumilus</i> , <i>B. licheniformis</i> , <i>B. firmus</i> , <i>B. atrophaeus</i> , <i>B. amylolyquefaciens</i> , <i>B. mojavensis</i> , <i>Lysinibacillus sphaericus</i> .	Phenotypic tests, 16S rRNA gene sequencing	Ghana	Amua-Awua et al. 2006; Meerak et al. 2008;
Dawadawa-type condiment	Bambara groundnut	<i>B. subtilis</i> subsp. <i>subtilis</i> , <i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> , <i>B. pumilus</i> and <i>B. licheniformis</i>	MALDI-TOF MS, 16S rRNA and <i>gyrA</i> genes sequencing	Ghana	Akanni et al. 2018
Bikalga	<i>Hibiscus sabdariffa</i>	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>B. cereus</i> , <i>B. pumilus</i> , <i>B. badius</i> , <i>Brevibacillus borstelensis</i> , <i>B. sphaericus</i> , <i>B. fusiformis</i>	Phenotypic tests, 16S-23S rRNA (ITS-PCR) gene sequencing	Burkina Faso	Bengaly 2001; Ouoba et al. 2008
Iru	<i>Parkia biglobosa</i> seed	<i>B. subtilis</i> , <i>B. amylolyquefaciens</i> , <i>B. cereus</i> , <i>B. licheniformis</i> , <i>B. pumilus</i> and <i>Brevibacillus formosus</i>	Phenotypic tests, ARDRA, ITS-PCR, ITS-PCR-RFLP, RAPD-PCR, PCR-DGGE, 16S rRNA gene sequencing	Nigeria	Adewumi et al. 2013; Adewumi et al. 2014
Tayohounta	Baobab seed	<i>B. licheniformis</i> , <i>B. pumilus</i> , <i>B. subtilis</i> , <i>B. thermoamylovorans</i>	PCR-DGGE and cloning of 16S rRNA PCR fragments	Benin	Chadare et al. 2011
Ogiri	Melon/Castor oil seeds	<i>B. safensis</i> , <i>B. siamensis</i> , <i>B. altitudinis</i> , <i>B. encimensis</i>	Phenotypic tests, 16S rRNA gene sequencing	Nigeria	Adeyemi et al. 2018; Odunfa 1985
Okpehe	<i>Prosopis africana</i> seeds	<i>B. subtilis</i> , <i>B. amylolyquefaciens</i> , <i>B. cereus</i> , <i>B. licheniformis</i>	Phenotyping, RAPD-PCR, ARDRA fingerprinting, 16S rRNA gene sequencing	Nigeria	Oguntoyinbo et al. 2010
Soumbala	<i>Parkia biglobosa</i> seed	<i>B. subtilis</i> , <i>B. pumilus</i> , <i>B. cereus</i> , <i>B. sphaericus</i> , <i>Brevibacillus borstelensis</i> , <i>B. thuringiensis</i> , <i>B. licheniformis</i> , <i>B. badius</i> , <i>Paenibacillus alvei</i> , <i>B. firmus</i> , <i>P. larvae</i> , <i>Brevibacillus laterosporus</i> , <i>B. megaterium</i> , <i>B. mycoides</i>	ITS-PCR, ITS-PCR RFLP, PFGE, 16S rRNA sequencing, RAPD-PCR fingerprint	Burkina Faso	Ouoba et al. 2004; Sarkar et al. 2002
Ugba	<i>Pentaclethra macrophylla</i>	<i>B. cereus</i> sensu lato, <i>Lysinibacillus xylanilyticus</i> , <i>B. clausii</i> , <i>B. licheniformis</i> , <i>B. subtilis</i> and <i>B. safensis</i>	Phenotyping, sequencing of 16S rRNA, <i>gyrB</i> and <i>rpoB</i> genes, 16S-23S rRNA ITS-PCR and rep-PCR	Nigeria	Ahaotu et al. 2013
Afitin, sonru and iru Nététou	<i>Parkia biglobosa</i> seed	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>B. cereus</i> ,	ITS-PCR-RFLP, 16S rRNA gene sequencing	Benin	Azokpota et al. 2007
Parkia biglobosa seed	<i>B. licheniformis</i> , <i>B. coagulans</i> , <i>B. subtilis</i> , <i>B. pumilus</i>	Phenotypic tests	Senegal	N'dir et al. 1994; N'dir et al. 1997	
Soy-daddawa	Soybean	<i>B. subtilis</i>	Phenotypic, PCR-DGGE and 16S rRNA gene sequencing	Nigeria	Ezekoli et al. 2016
Owoh	cotton seeds (<i>Gossypium hirsutum</i>)	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>B. pumilus</i> ,	Phenotypic tests	Nigeria	Sanni and Ogbonna 1991; Ezekiel et al. 2015
Maari	Baobab seed	<i>B. subtilis</i> , <i>B. licheniformis</i> , <i>B. velezensis</i> , <i>B. safensis</i> , <i>B. megaterium</i> , <i>B. endophyticus</i> , <i>B. cereus</i> , <i>B. coagulans</i> , <i>B. circulans</i>	Phenotypic tests, rep-PCR (GTG)-fingerprinting and 16S rRNA gene sequencing	Burkina Faso	Parkouda et al. 2010
Soumbara	<i>Parkia biglobosa</i> seed	<i>B. subtilis</i> , <i>B. velezensis</i> , <i>B. pumilus</i>	16S rRNA genes sequencing, RFLP analysis	Côte d'Ivoire	Adjoumani et al. 2019
Mbuja	<i>Hibiscus sabdariffa</i>	<i>B. subtilis</i> , <i>B. megaterium</i> , <i>B. amylolyquefaciens</i> , <i>B. pumilus</i> and <i>B. cereus</i>	Phenotypic tests, 16S rRNA and <i>gyrB</i> genes sequencing	Cameroon	Mohamadou et al. 2013
Yanyanku	<i>Hibiscus sabdariffa</i>	<i>B. subtilis</i> , <i>B. cereus</i> , <i>B. amylolyquefaciens</i> , <i>B. licheniformis</i> , <i>B. safensis</i> , <i>B. altitudinis</i> , <i>B. aryabhattachai</i> , <i>B. flexus</i> , <i>B. circulans</i>	Phenotypic tests, rep-PCR, M13-PCR, 16S rRNA, <i>gyrA</i> , <i>gyrB</i> sequencing	Benin	Agbobatinkpo et al. 2013
Ikpiru	<i>Hibiscus sabdariffa</i>	<i>B. subtilis</i> , <i>B. cereus</i> , <i>B. amylolyquefaciens</i> , <i>B. licheniformis</i> , <i>B. safensis</i> , <i>B. altitudinis</i> , <i>B. aryabhattachai</i> , <i>B. flexus</i> , <i>B. circulans</i>	Phenotypic tests, rep-PCR, M13-PCR, 16S rRNA, <i>gyrA</i> and <i>gyrB</i> genes sequencing	Benin	Agbobatinkpo et al. 2013
Kantong	Kapok tree (<i>Ceiba pentandra</i>)	<i>B. subtilis</i> subsp. <i>subtilis</i> , <i>B. safensis</i> , <i>B. amylolyquefaciens</i> subsp. <i>plantarum</i>	Phenotypic tests, M13-PCR typing, 16S rRNA and <i>gyrA</i> genes sequencing	Ghana	KpiKpi et al. 2014



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Technologically relevant *Bacillus* species and microbial safety of West African traditional alkaline fermented seed condiments

James Owusu-Kwarteng, Charles Parkouda, Gbenga Adedeji Adewumi, Labia Irène Ivette Ouoba & Lene Jespersen

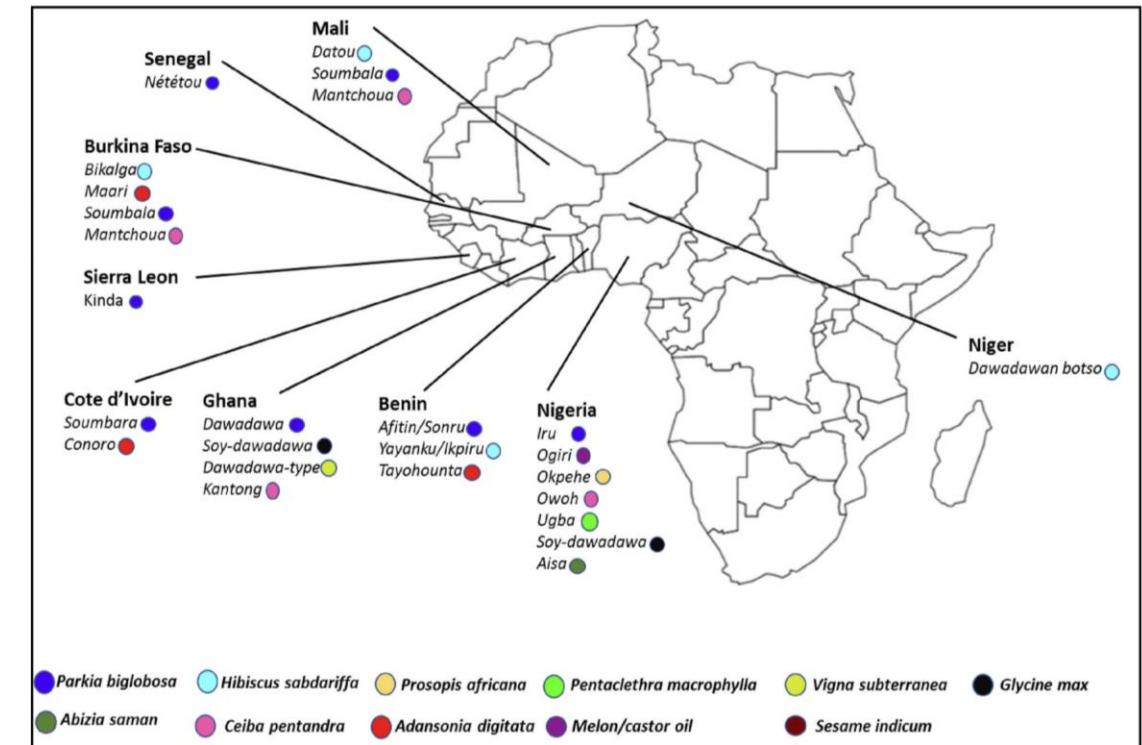
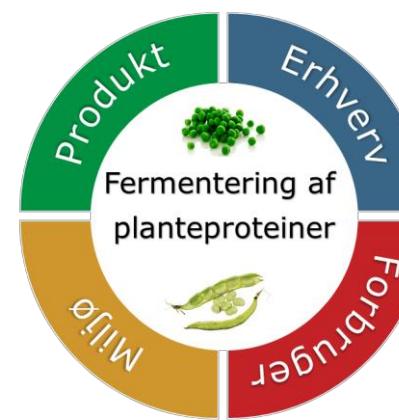


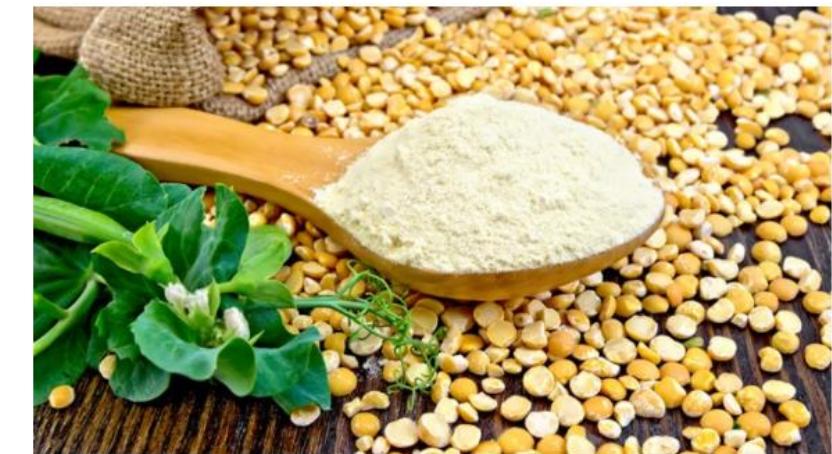
Figure 1. Common plant species for the processing of West African fermented seed condiments.

Fermentation of plant based raw materials – needs novel starter and bio-protective cultures

- Fermentation for production of next generation proteins requires:
 - Enhanced taste (umami), texture and nutrient composition
 - Masking of off-flavours from e.g. pea and beans
 - Bio-preservation of microbial pathogens and spoilers



Ærter, hestebønner og græs kan blive velsmagende alternativer til kød



Ærter har før været en naturlig del af hverdagskosten i Danmark. Nu kan de få en renæssance som kilde til protein i nye produkter, der erstatter kød. Foto: Colourbox

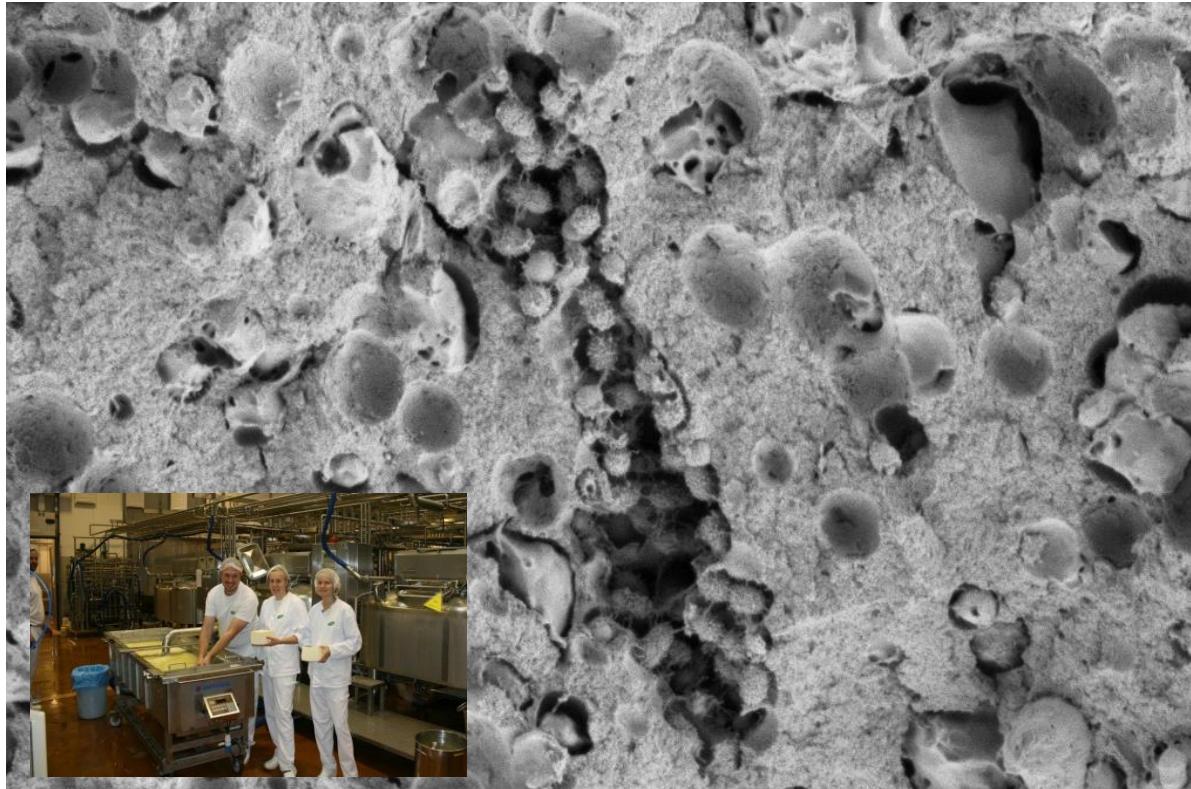
Ny fermenteringsteknologi, som forbedrer smag, tekstur og næringsværdi, kan gøre ærter, hestebønner, kartofler og græs til endnu mere attraktive proteinkilder i plantebaserede fødevarer.

Efterspørgslen på plantebaserede produkter stiger støt. Mange forbrugere ønsker at spise mindre kød, fordi de i stigende grad er opmærksomme på fødevarernes klimabelastning, men de nye plantebaserede alternativer lever ikke altid op til forventningerne.

"Der er allerede en del kødalternativer på markedet, men forbrugerundersøgelser viser, at mange ønsker et større udvalg og mere velsmagende produkter," siger Eleonora Miquel Becker fra Teknologisk Institut, som står i spidsen for GUDP-projektet FERMPRO.

The challenge of finding good starter cultures for new plant-based food

We have extensive knowledge on cultures for milk-based products – but what about plant-based products?



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EHT = 3.00 kV Signal A = SE2
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Fermentation can:

- improve taste and texture
- enhance bioavailability of nutrients
- optimise shelf life and safety

Plant materials are:

- very diverse, complex, etc.
- very different from e.g. milk matrices

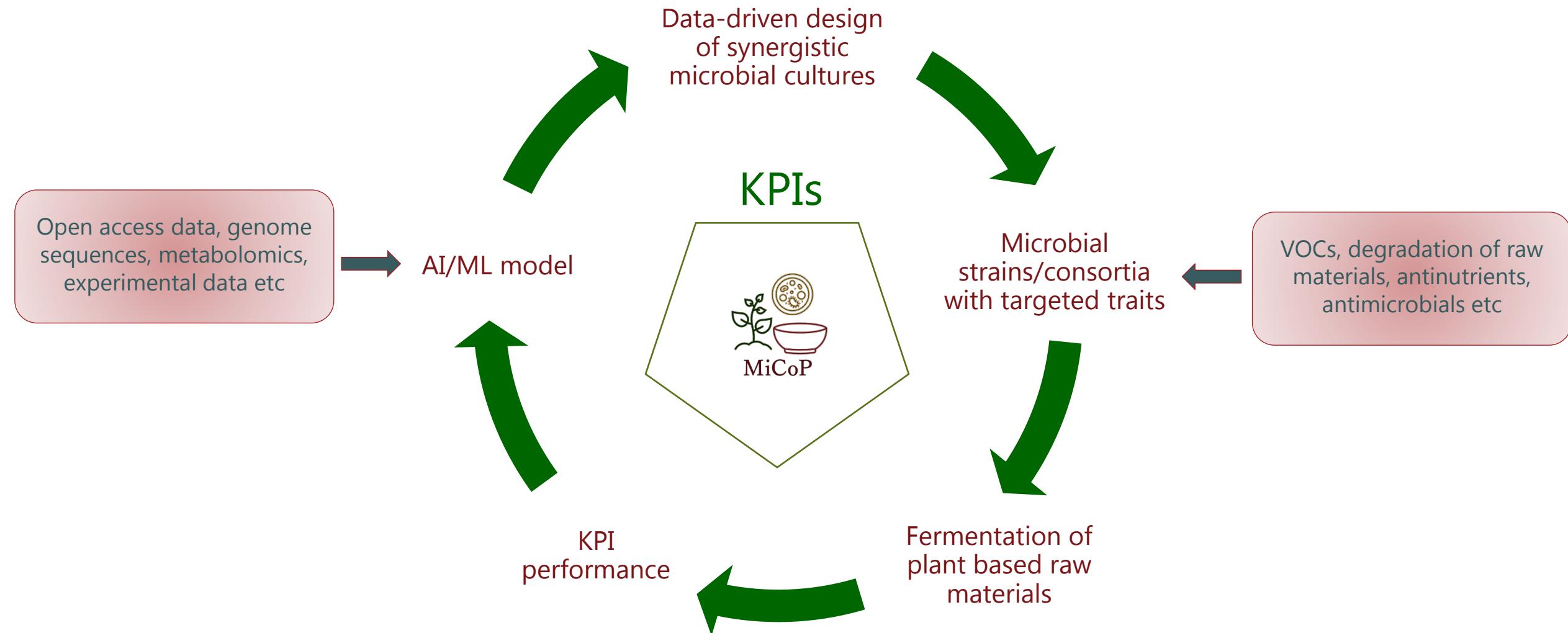
Fermentation of plants requires detailed knowledge on:

- how microbes interact with plant cells
- enzymes required for degradation of plant cells
- best cultures and practices for optimised flavour, nutritional properties and shelf life

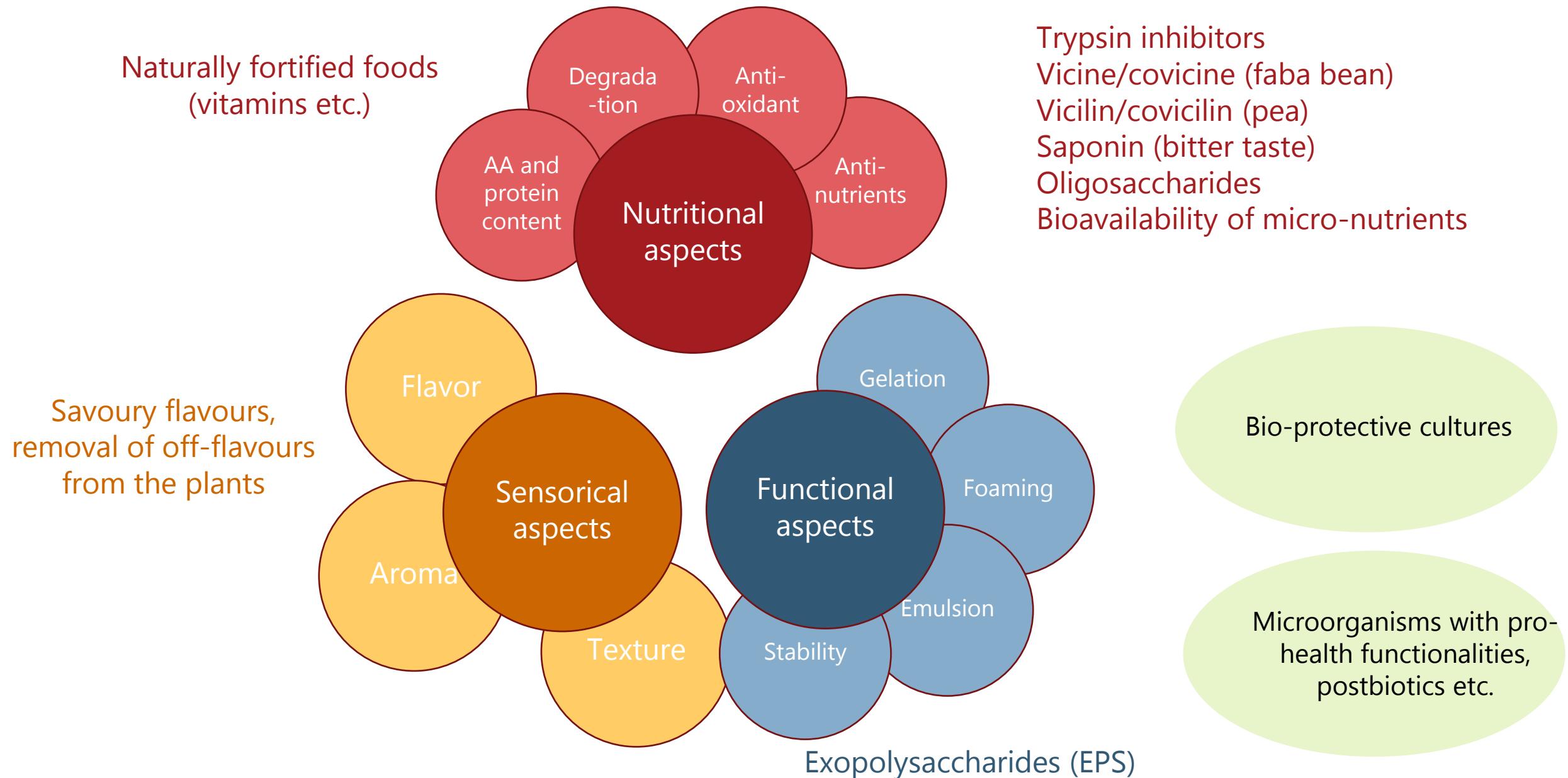
TRIAL AND ~~ERROR~~

Use of AI/ML for identifying and optimizing microbial KPIs

Key Performance Indicators for microbial fermentation abilities



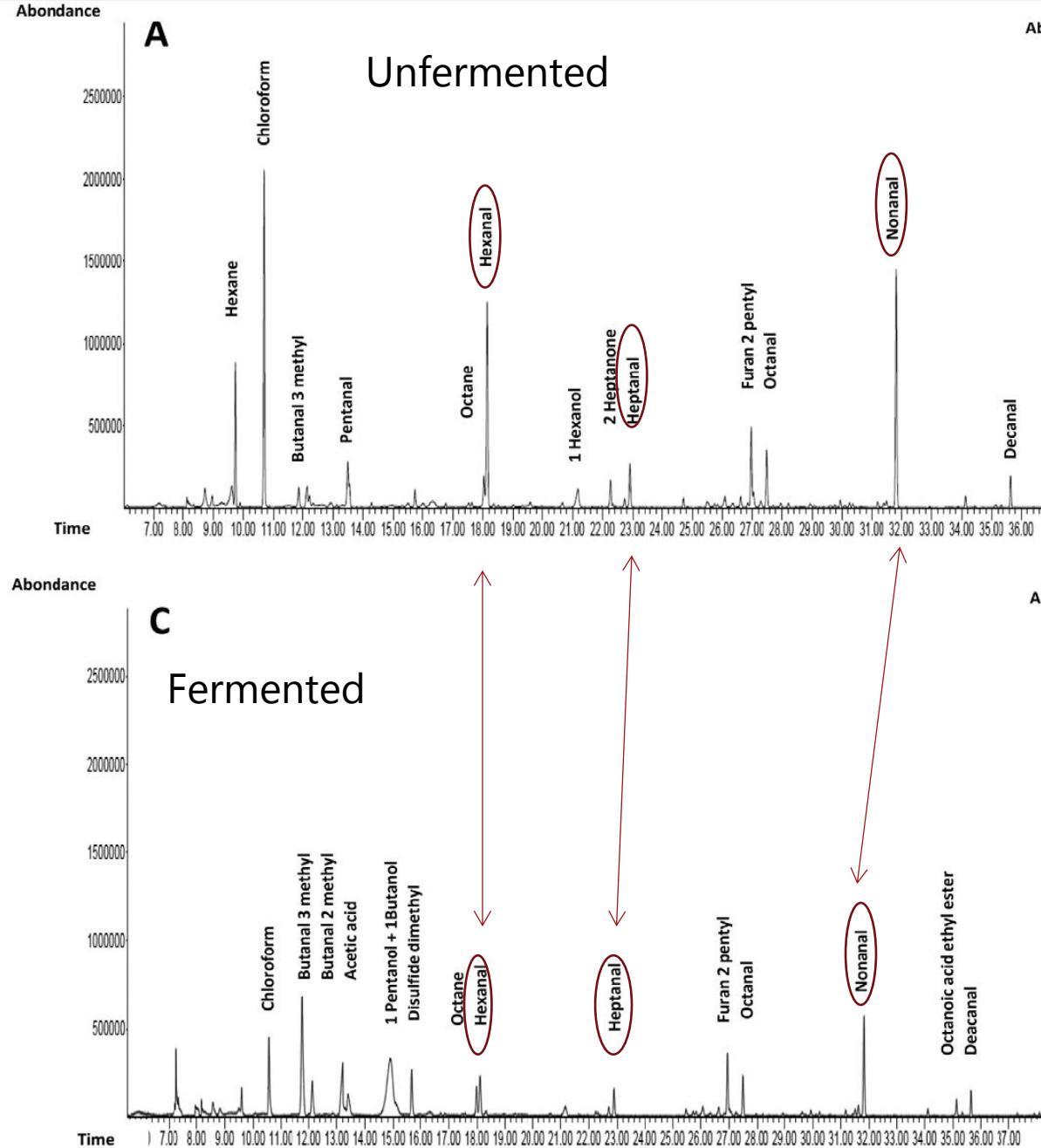
Possibilities with fermentation (plant-based foods)



Protein source	Protein type	Sensorical aspects				Nutritional aspects				Functional aspects				Microorganisms			Literature survey
		Aroma	Sensory analysis	Nucleotides	Off-flavor	Texture	AA & protein content	Degradation / IVPD	Anti-nutritionals	Antioxidant	Gelation	Foaming	Stability	Emulsify	LAB	Yeast	Mold
Pea	WF	•	•		•									•	•	•	Ben Harb et al. 2019
Pea	WF	•			•									•	•		Youssef et al. 2019
Pea	WF	•	•		•		•	•	•		•		•	•	•		Arteaga et al. 2021
Pea	WF	•			•							•	•	•	•		Schindler et al. 2017
Pea	WF													•			Stanisavljevic et al. 2015
Pea	DF						•	•	•	•				•			Coda et al. 2014
Pea	DF						•	•	•	•				•			Çabuk et al. 2018a
Pea	DF						•				•	•	•	•	•		Çabuk et al. 2018b
Pea	DF						•	•	•						•		Kumitch et al., 2019
Pea	W		•	•											•		Mouritsen et al. 2017
Faba	WF						•	•	•	•				•			Verni et al., 2019
Faba	WF						•	•	•	•					•		Polanowska et al. 2020
Faba	DF				•		•	•	•					•			Xu et al., 2019
Faba	DF						•	•	•	•				•			Rosa-Sibakov et al., 2018
Faba	DF						•	•	•	•				•			Rizello et al., 2019

Different microorganisms give different sensory attributes

Sensorical aspects



Removal of off-flavours

Volatile compounds (off-flavors) in V10

- In general, a significant increase in the number and percentage of VOCs was obtained after fermentation.
- Different compounds, variously comprising acids, alcohols, aldehydes, esters, and ketones, appeared or strongly arose in fermented V10
- Degradation of off-flavors (hexanal, heptanal, nonanal)

Japanese sake is the beverage with the most pronounced umami flavour



Addition of koji to steamed rice



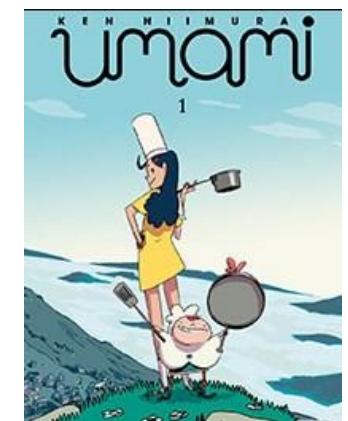
Sake fermenters



Koji with *Aspergillus oryzae*



Fermenter with sake-yeast (*S. cerevisiae*)



From Koji to oysters and champagne – umami makes it!



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scientific reports

OPEN Umami synergy as the scientific principle behind taste-pairing champagne and oysters

Charlotte Vinther Schmidt, Karsten Olsen & Ole G. Mouritsen

Check for updates

Research Article

Received: 11 October 2014 Revised: 15 December 2014 Accepted article published: 26 December 2014 Published online in Wiley Online Library: 19 January 2015
(wileyonlinelibrary.com) DOI 10.1002/jfa.7058

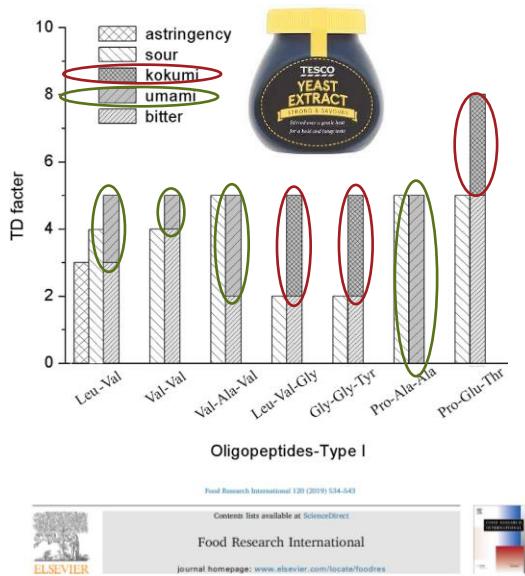
Discovery of kokumi peptide from yeast extract by LC-Q-TOF-MS/MS and sensomics approach

Jianbin Liu,^a Huanlu Song,^{a*} Ye Liu,^a Pei Li,^b Juan Yao^b and Jian Xiong^b

Abstract

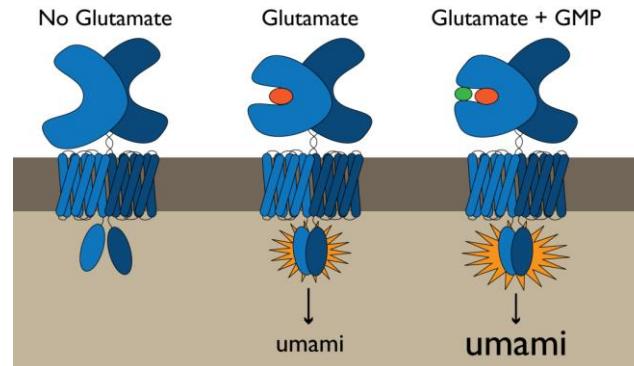
BACKGROUND: Yeast extract can impart thickness, complexity and long-lasting taste impression, coined kokumi taste, to blank chicken broth. In this research, the kokumi-active peptide in yeast extract was discovered by ultrafiltration, liquid chromatographic and quadrupole-time-of-flight-tandem mass spectrometric technologies. Furthermore, the sensory characters of these peptides were evaluated by a sensomics approach.

Yeasts give the taste

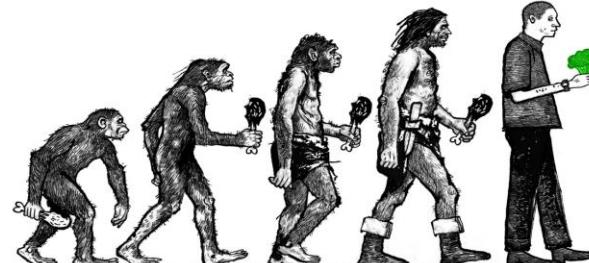
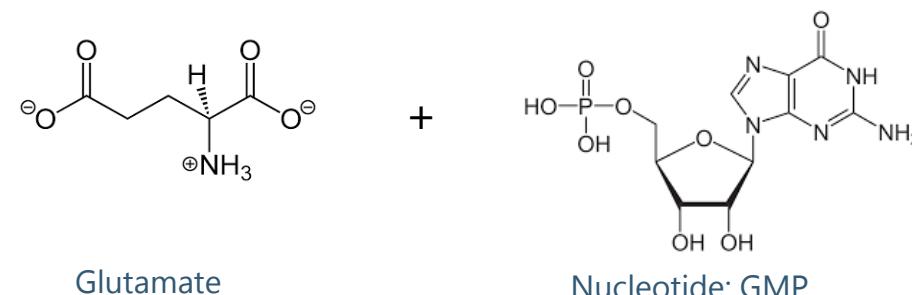


umami: 'pleasant savoury taste'

Monosodium γ -glutamate (MSG) (+ umami peptides) sensation is enhanced by 5'- ribonucleotides



Model of the T1R1/T1R3 umami taste receptor



Kokumi: 'mouthfulness'

Mainly γ -glutamyl - and leucyl peptides

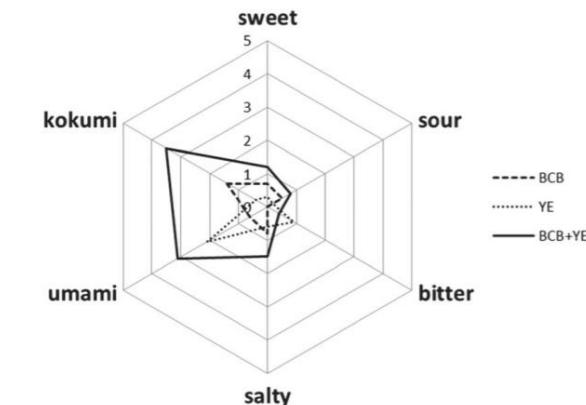


Figure 1. Taste profile of blank chicken broth (BCB), yeast extract (YE) and the mixture (BCB + YE).

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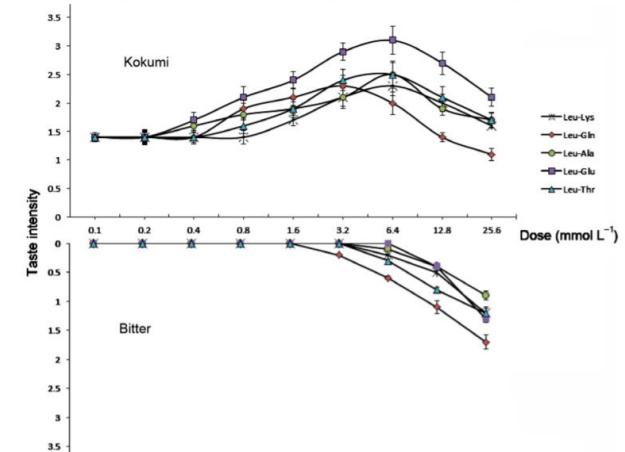


Figure 6. Dose – response sensory evaluation of the five leucyl dipeptides identified in yeast extract.



Functionality of *Bacillus* spp. in food fermentation

Technological property	Specific activity
Aroma and flavour development	✓ Umami (meat-like sensation), small peptides, volatile compounds
Bioconversion of plant components	✓ Enhanced digestibility and softening of textures ✓ Increase in free amino acids and peptides ✓ Degradation of non-digestible carbohydrates (poly- and oligosaccharides) ✓ Prevention of flatulence ✓ Structural changes and production of surfactants
Enhanced enzymatic activities	✓ Proteolysis (amino acids, peptides, ammonia) ✓ Lipolysis (FFA)
Microbial interactions	✓ Antagonism against <i>B. cereus</i> , <i>Aspergillus ochraceus</i> and pathogenic bacteria (<i>Salmonella Typhimurium</i> , <i>Yersinia enterocolitica</i> etc.)
Antimicrobial peptides (AMPs)	✓ Subtilin (AIP, QS) and other bacteriocins ✓ Lipopeptides (growth inhibition of pathogens)

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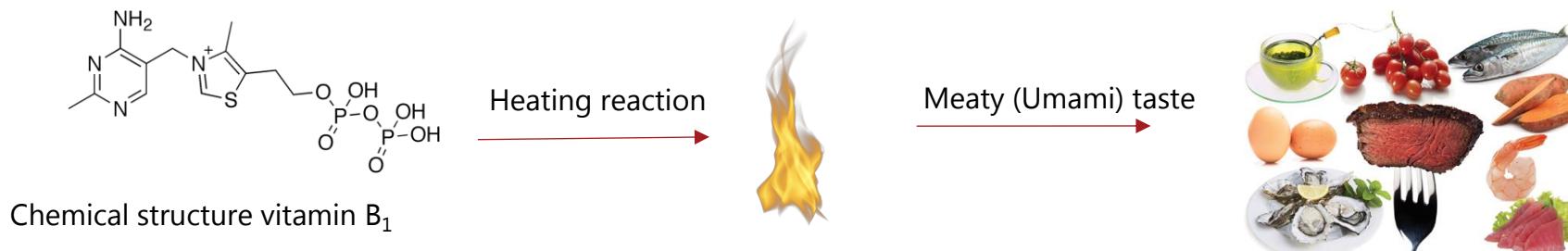
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What can *Bacillus* spp. offer to plant based food?

Importance of flavour precursors (incl. vitamin B₁), peptides and enhanced vitamin B₁₂ synthesis

Flavour precursor → meaty taste in heating reaction

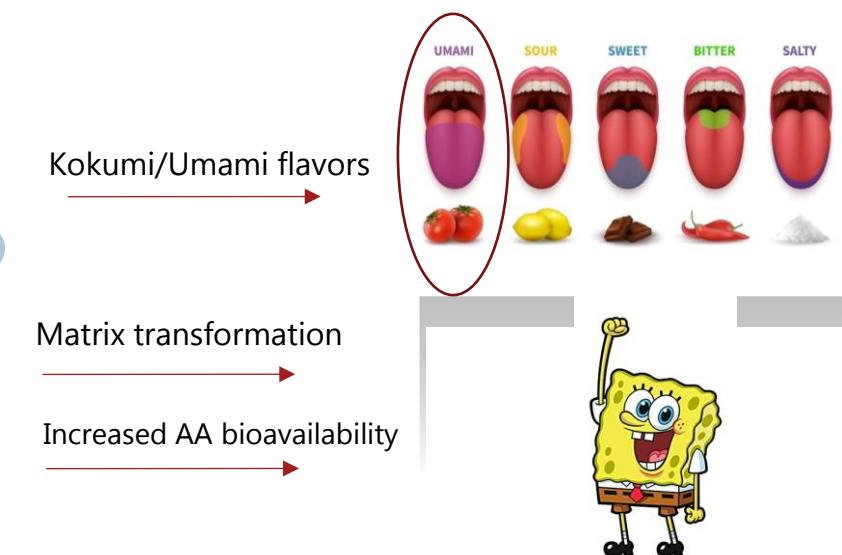
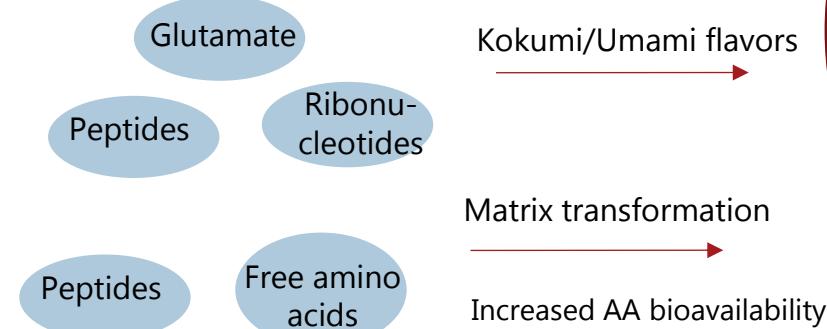


Chemical structure vitamin B₁

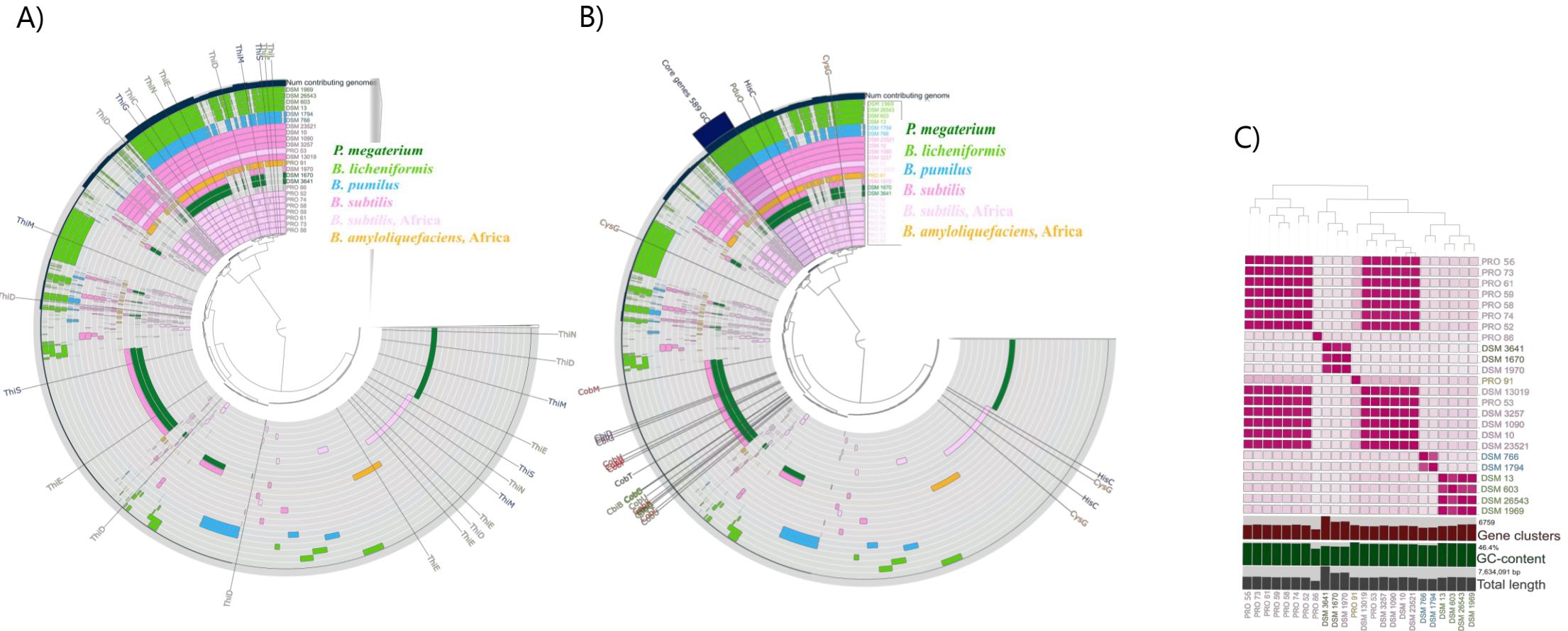
Importance of protease activity for plant based food production



Plant protein hydrolysis



Vitamin production by *Bacillus* spp. Genome sequencing of sub-Saharan *Bacillus* isolates



Pangenomic analysis of 24 *Bacillus* isolates, identifying genes for A) vitamin B₁ biosynthesis, and B) vitamin B₁₂, and C) Average Nucleotide Identity (ANI) heatmap ranging from 70-100 % similarity, genome GC content, genome length in bp, and number of gene clusters (GC).

Yeasts for bio-fortification and improved bioavailability of divalent ions of cereals

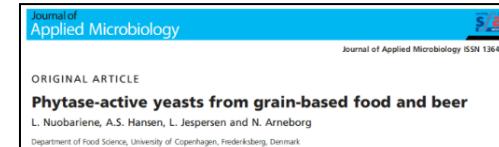
Phytase activity



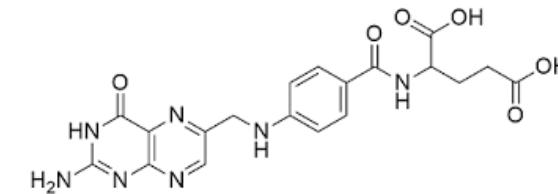
Phytic acid

Species	Strains	Phytase activities*	
		mU ml⁻¹	mU (10¹⁰ CFU)⁻¹
<i>S. cerevisiae</i>	ATCC26108	9 ± 1	281 ± 22
	DGI342	10 ± 1	62 ± 7
	CBS1236	3 ± 1	28 ± 2
	P10	28 ± 1	457 ± 52
	KVL013	3 ± 1	83 ± 28
	KVL015	67 ± 1	732 ± 8
<i>S. pastorianus</i>	KVL008	76 ± 6	1981 ± 20
	KVL016	3 ± 1	26 ± 3
<i>C. krusei</i>	K132	30 ± 5	509 ± 62
	K204	20 ± 3	111 ± 18
	P1	14 ± 2	110 ± 5
	P2	50 ± 14	595 ± 58
	P11	35 ± 3	460 ± 53

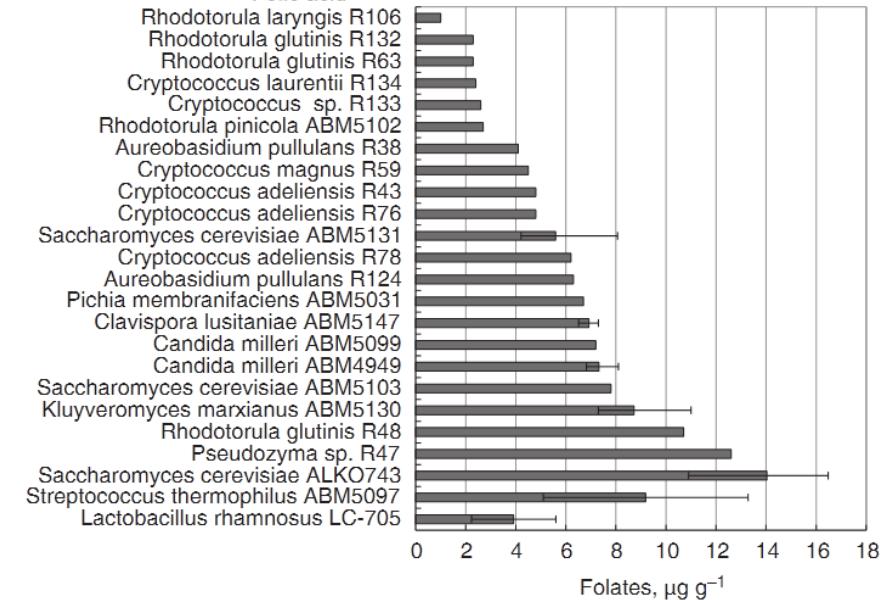
Functional properties are species and strain dependent



Vitamin B₉ (folate) production



Folic acid



Important species encounter food borne yeasts as *S. cerevisiae* and *C. krusei* (*P. kudriavzevii*)

Important species encounter food borne yeasts as *S. cerevisiae* and *K. marxianus*

Fermentation degraded anti-nutrients: α -galactosides in faba beans

- α -galactosides of sucrose are fermented by the intestinal microbiota causing gastrointestinal disorders, i.e. verbascose, stachyose and raffinose
- Fermentation with *Lactiplantibacillus plantarum* enabled the complete or partial degradation of verbascose, stachyose and raffinose.

Table 3

Sugars concentration (g/kg) in faba bean doughs before and after 48 h of fermentation at 30 °C with *Lb. plantarum* DPPMAB24W. The list of the abbreviations for faba bean accessions is reported in Table 1.

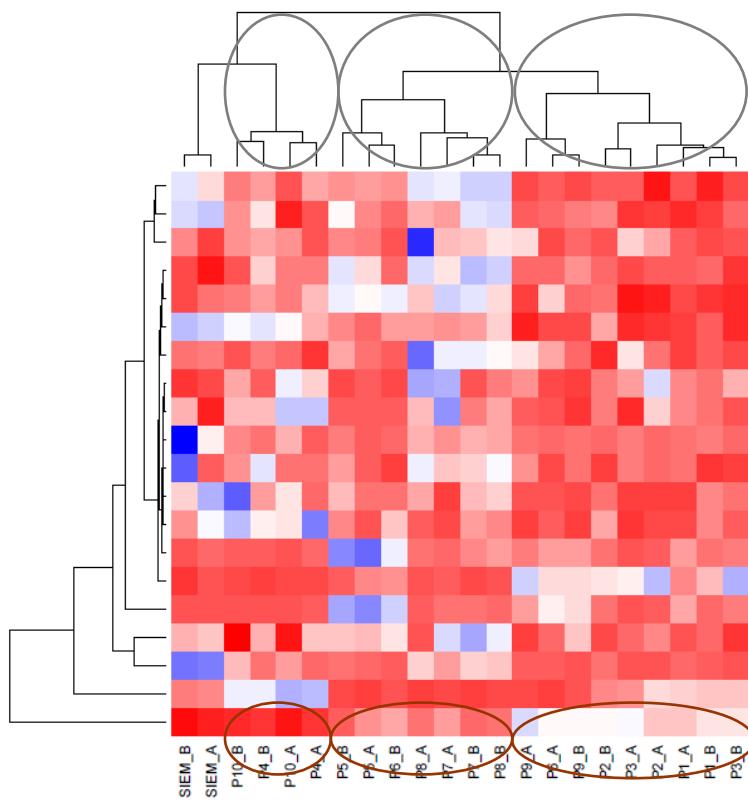
	Verbascose		Stachyose		Raffinose		Sucrose		Fructose	
	T ₀	T ₄₈	T ₀	T ₄₈	T ₀	T ₄₈	T ₀	T ₄₈	T ₀	T ₄₈
F1	38.7 ± 2.1 ^{cd}	2.0 ± 0.1 ^b	27.2 ± 1.5 ^b	16.7 ± 0.9 ^b	6.1 ± 0.3 ^c	5.1 ± 0.3 ^{ab}	19.4 ± 1.0 ^{bc}	16.4 ± 0.5 ^d	Nd	0.4 ± 0.0 ^{ab}
F2	51.2 ± 2.9 ^b	0.2 ± 0.0 ^d	11.6 ± 0.7 ^c	0.3 ± 0.0 ^d	16.4 ± 0.7 ^b	0.5 ± 0.0 ^d	19.2 ± 1.1 ^{bc}	23.8 ± 1.8 ^{bc}	5.2 ± 0.2 ^a	nd
F3	33.7 ± 1.8 ^d	1.1 ± 0.1 ^c	11.8 ± 0.7 ^c	0.5 ± 0.0 ^d	1.7 ± 0.1 ^e	0.1 ± 0.0 ^d	15.2 ± 0.8 ^c	19.4 ± 0.8 ^c	3.8 ± 0.1 ^b	nd
F4	20.0 ± 1.3 ^f	0.9 ± 0.0 ^c	6.3 ± 0.4 ^e	0.2 ± 0.0 ^d	0.5 ± 0.0 ^f	nd	11.0 ± 0.4 ^d	16.0 ± 0.5 ^d	5.7 ± 0.3 ^a	nd
F5	42.4 ± 2.1 ^c	nd	25.7 ± 1.5 ^b	5.8 ± 0.5 ^c	3.8 ± 0.2 ^d	nd	37.1 ± 2.3 ^a	48.2 ± 2.7 ^a	Nd	0.2 ± 0.0 ^b
F6	36.3 ± 1.8 ^{cd}	0.1 ± 0.0 ^d	7.8 ± 0.5 ^d	0.1 ± 0.0 ^d	2.3 ± 0.2 ^d	0.2 ± 0.0 ^d	23.8 ± 1.4 ^b	18.6 ± 0.6 ^c	4.1 ± 0.1 ^{ab}	nd
F7	36.3 ± 1.6 ^{cd}	nd	9.5 ± 0.6 ^c	0.2 ± 0.0 ^c	1.5 ± 0.2 ^e	0.3 ± 0.0 ^d	24.9 ± 1.4 ^b	18.1 ± 0.7 ^c	1.3 ± nd ^d	0.4 ± 0.0 ^{ab}
F8	67.9 ± 4.6 ^a	8.84 ± 0.6 ^a	38.1 ± 2.4 ^a	16.9 ± 0.9 ^a	16.7 ± 1.1 ^a	nd	32.7 ± 2.8 ^a	19.3 ± 1.7 ^{bc}	Nd	0.6 ± 0.0 ^a
F9	21.3 ± 1.4 ^f	nd	8.1 ± 0.5 ^d	0.1 ± 0.0 ^d	5.0 ± 0.6 ^{cd}	4.3 ± 0.2 ^b	22.3 ± 1.4 ^b	16.8 ± 0.6 ^{cd}	4.0 ± 0.1 ^{ab}	nd
F10	18.3 ± 1.2 ^f	nd	1nd ± 0.7 ^c	nd	7.6 ± 0.4 ^c	5.9 ± 0.2 ^{ab}	37.5 ± 2.1 ^a	26.8 ± 1.9 ^b	4.9 ± 0.2 ^a	nd
F11	17.1 ± 1.0 ^f	nd	5.7 ± 0.4 ^e	nd	2.2 ± 0.2 ^d	nd	22.7 ± 1.1 ^b	15.0 ± 0.4 ^d	1.8 ± 0.2 ^{cd}	0.4 ± 0.0 ^{ab}
F12	43.0 ± 2.7 ^c	nd	13.1 ± 0.8 ^c	3.6 ± 0.6 ^c	22.7 ± 1.0 ^a	7.5 ± 0.4 ^a	37.8 ± 2.3 ^a	27.5 ± 2.4 ^b	2.3 ± 0.1 ^c	nd
F13	26.4 ± 1.7 ^e	nd	8.4 ± 0.6 ^d	0.1 ± 0.0 ^d	3.9 ± 0.3 ^d	2.8 ± 0.1 ^c	22.3 ± 1.2 ^b	11.0 ± 0.1 ^e	2.4 ± 0.2 ^c	nd

The data are the means of three independent experiments ± standard deviations (n = 3).

a-f Values in the same column, with different superscript letters, differ significantly (P < 0.05).

nd: not detected.

From bio-waste to functional ingredients



Positive in relation to obesity/diabetes

1
Relative abundance of OTU

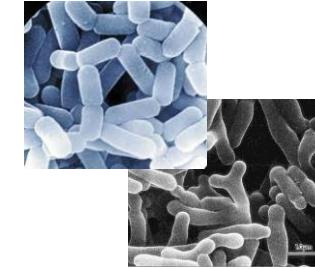
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20 OTU/genera spp. (48 h)

Lower predominance

o_Clostridiales_f_g_s
 o_Clostridiales_f_Ruminococcaceae_g_Ruminococcus_s
 o_Bacteroidales_f_Bacteroidaceae_g_Bacteroides_s
 o_Clostridiales_f_Ruminococcaceae_g_Oscillospira_s
 o_Clostridiales_f_Lachnospiraceae_g_Coprococcus_s
 o_Clostridiales_f_Lachnospiraceae_g_Dorea_s
 o_Bacteroidales_f_g_s
 o_Clostridiales_f_Veillonellaceae_g_Phascolarctobacterium_s
 o_Clostridiales_f_Clostridiaceae_g_Clostridium_s
 o_Bacteroidales_f_S24-7_g_s
 o_Bacteroidales_f_Bacteroidaceae_g_Bacteroides_s_uniformis
 o_Clostridiales_f_Lachnospiraceae_g_Blautia_s
 o_Clostridiales_f_Lachnospiraceae_g_[Ruminococcus]_s_gnavus
 o_Clostridiales_f_Lachnospiraceae_g_Butyryvibrio_s
 o_Clostridiales_f_Ruminococcaceae_g_Faecalibacterium_s_prausnitzii
 o_Clostridiales_f_Lachnospiraceae_g_Lachnospira_s
 o_Bacteroidales_f_Prevotellaceae_g_Prevotella_s
 o_Clostridiales_f_Lachnospiraceae_g_s
 o_Bacteroidales_f_Prevotellaceae_g_Prevotella_s_copri
 o_Clostridiales_f_Ruminococcaceae_g_s

High predominance



CP Kelco

KMC
LET'S TAKE FOOD FORWARD

CHR HANSEN

Improving food & health



- The abundance of specific bacterial genera in the gut was affected by pectin fermentation
- The main structural features of pectins related to the shaping of the gut microbiota included degree of esterification, content of monosaccharides, degree of branching and content of homogalacturonan/rhamnogalacturonan
- Pectin interventions can be used for targeting specific bacterial populations and, thereby, for beneficial modulation of the gut microbiota

Are fermented plant-based foods always safe?

- Food safety still has to be respected, also in plant based foods!
- Potential safety issues:
 - Production of toxins (bacterial, mycotoxins), biogenic amines
 - Survival or growth of infectious microorganisms
 - Natural toxic compounds
- Solution:
 - Starter cultures
 - Controlled process conditions
 - Knowing your raw materials and potential hazards associated with them

5 minutes of reflection with your fellow students on how fermentation can be used to enhance the quality and sustainability of new innovative fermented plant-based products



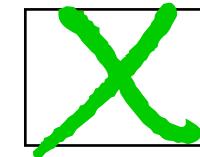
Prepare for discussion in Plenum

Take home messages

- Plant-based fermentations of food and beverages are abundant world-wide
- Plant-based fermentations can be adopted for improving sensorial-, nutritional- and functional aspects, e.g. umami, B₁₂ vitamin, lower amount of phytic acid, lower amount of α-galactosides etc.
- Different groups of microorganisms have different functionalities in plant-based food and beverages and might differ from those used in animal-based foods as yoghurt, cheese etc.
- Plant-based fermented foods are sustainable, might impact your health and can shape your gut microbiota
- Specific bio-protective cultures targeting plant-based products need to be developed.
- As for all fermentations, food safety has to be respected

Can microorganisms add in creating a more sustainable and healthy food production?

YES



NO



Thank you for your attention