Developments in Cheese Starter Technology

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DuPont Nutrition & Health

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Using Starter cultures

The practicality of employing cultures in cheese making has been tested.

Upon the belief that lactic acid bacteria are the main agents in the curing of cheese this species has been suggested as the “Starter” under the following conditions:

- The organism should be a pure lactic germ
- Free from undesirable aroma
- Adapted for vigorous development in milk

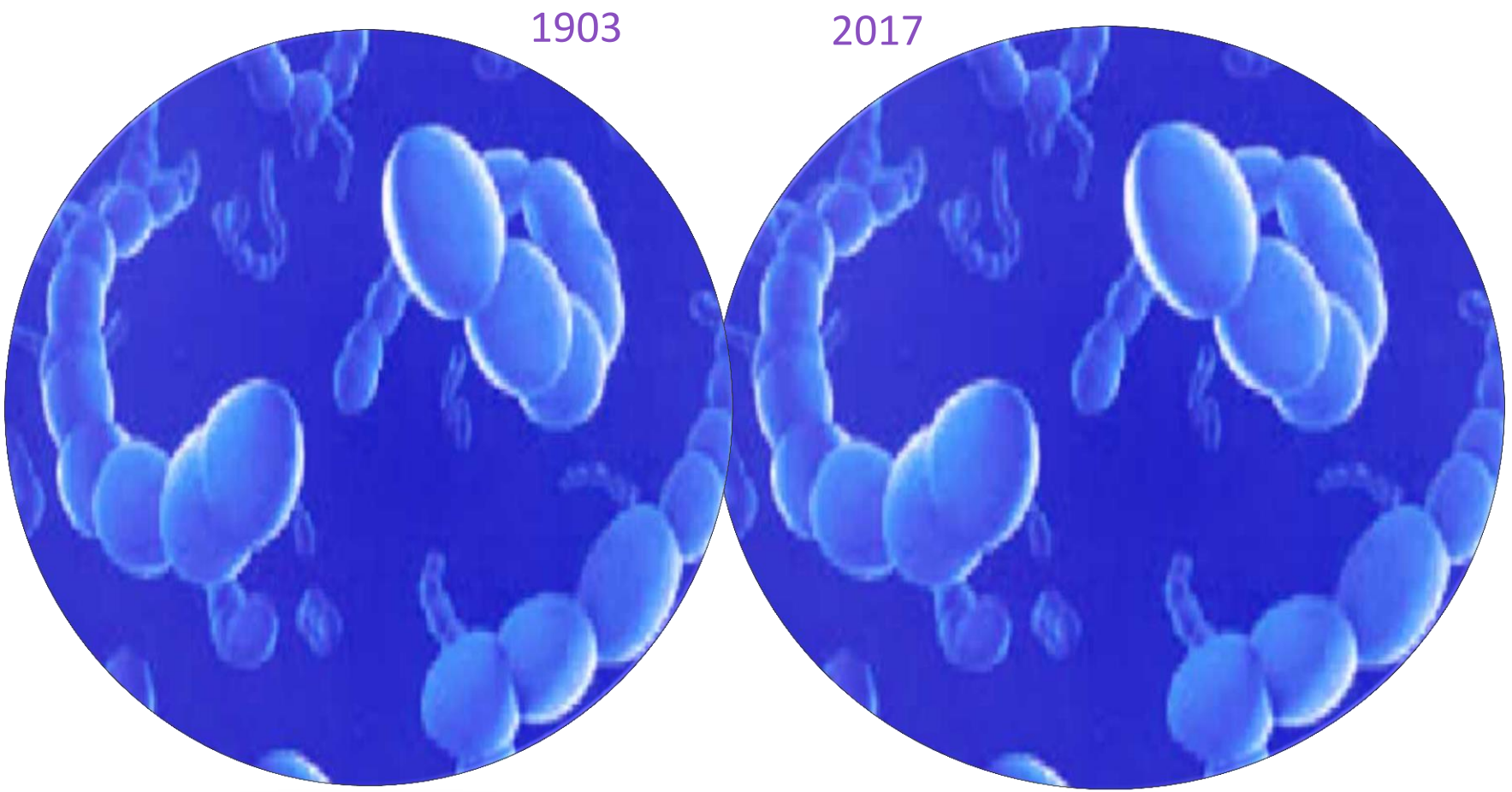
<u>The advantages accruing from the use of such a “Starter”</u>

- Saves time in the process of manufacture
- Aids development of a proper amount of acid for a typical cheese
- The flavour and quality of cheese is preferable to that which has not been thus produced

Bacteriology of Milk 1903 by Harold Swithinbank

Lactococcus lactis

Magnification 20,000X. Scanning electron micrograph



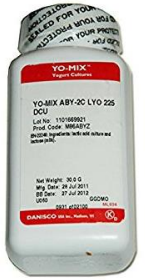
Physical forms of starter cultures

- Daily Propagation
- Frozen Pints
- Immediate Inoculation to the bulk starter tank
  - Your starter for 1000
- Direct Vat Inoculation
  - Frozen cans
  - Frozen pellets
  - Freeze dried















Lactic Acid culture collections

Name	Category	Description
MA, MM & BT Series PROBAT 505	•*Heterofermentative defined mesophilic* •*Heterofermentative Undefined mesophilic*	•*Lactococcus lactis subsp lactis (O culture)* •*Lactococcus lactis subsp cremoris(O culture)* •*Lactococcus lactis subsp lactis biovar diacetylactis (D culture)* • *Leuconostoc species (L culture)*
RA series MR & MRF series	•*Defined Homofermentative meso&thermo* •*Heterofermentative meso&thermo*	•*Lactococcus lactis subsp lactis (O culture)* •*Lactococcus lactis subsp cremoris(O culture)* •*Lactococcus lactis subsp lactis biovar diacetylactis (D culture)* •*Streptococcus thermophilus*
RM & RAM Series PROBAT 505	•*Defined Homofermentative meso&thermo* •*Heterofermentative meso&thermo* •*Heterofermentative Undefined mesophilic*	•*Lactococcus lactis subsp lactis (O culture)* •*Lactococcus lactis subsp cremoris(O culture)* •*Lactococcus lactis subsp lactis biovar diacetylactis (D culture)* •*Streptococcus thermophilus*
TM series TA Series STAR series	•*Streptococcus thermophilus* •*Lb. Helveticus & Other Lactobacilli species*	•*Streptococcus thermophilus* •*Lactobacillus helveticus* •*Lactobacillus bulgaricus*
TA 50 series STAM series	•*Streptococcus thermophilus*	•*Streptococcus thermophilus*
TA 60 series	•*Streptococcus thermophilus*	•*Streptococcus thermophilus*

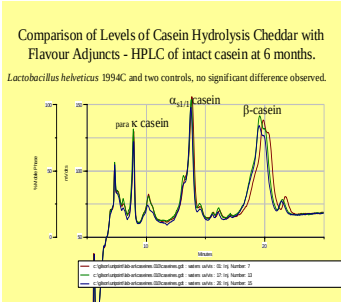
Ripening Cultures





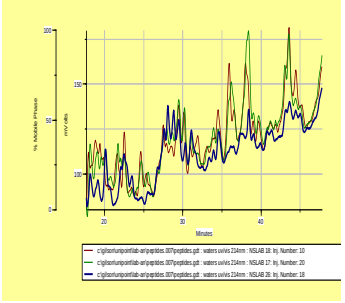
Product	Composition	Application
CB2	Penicillium roqueforti	Strong Blue Tast - Blue Green
KL71	Kluyveromyces lactis	Flavour in soft cheese and hole formation
Linens W	Brevibacterium linens,	Orange colour Aroma, fast growth
MGE	Arthobacter nicotianae	Strong aminopeptidase activity, fast growth
MVA	Staphylococcus xylosus	Stimulation of lactic acid bacteria
Mycodore	Trichothecium domesticum	Saint Nectaire Aspect
OFR 20	Brevibacterium casei, Brevibacterium linens, Debaromyces hansenii, Candid utils	Blend for colouration of smear cheese
OFR 9	Brevibacterium casei, Brevibacterium linens, Candida utils, Geothricium candidum	Appearance and flavour of all the surface ripening
PA	Penicillium roqueforti	Mild Blue Taste - Dark Green
PC 02	Penicillium Candidum	Ultra Filtered and stabilised cheese
PC 33	Penicillium Candidum	Anti Mucor activity, use every day
PC 53	Penicillium Candidum	Stabilised and Traditional
PC 12	Penicillium Candidum	Stabilised Cheese, Longer Shelf life
PC 42	Penicillium Candidum	Traditional cheese, Normal shelf life
PJ	Penicillium roqueforti	Typical Blue Taste - Middle Green
PLA	Brevibacterium linens, Geotrichum candidum, Debaromyces hensenii, Arthrobacter nicotianae	Complex blend for the appearance and flavour of the main French cheese
PV	Penicillium roqueforti	Strong Blue Taste - Blue Green
R2R	Rhodosporidium infirmominiatum	Flavour and colour in mixed smear cheese
	Debaromyces hansenii	Neutralisation for mix and sear cheese

Into the pathways of ripening in Flavour Creation



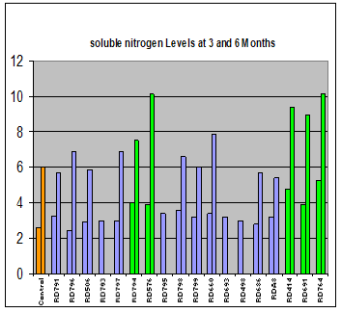
Casein Hydrolysis

Quantified by HPLC of cheese protein and rates of β-casein breakdown in buffered solution.



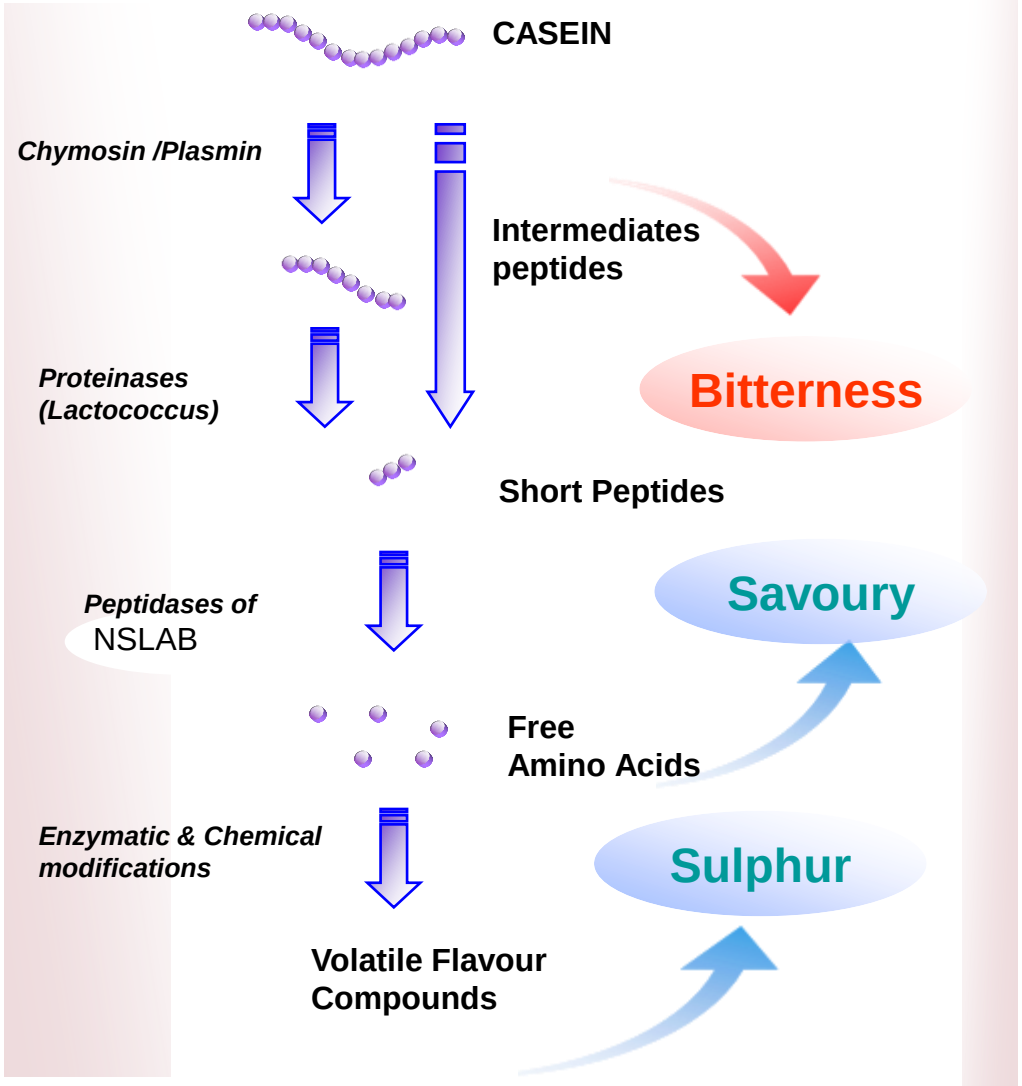
Peptide Hydrolysis

Quantified by HPLC of soluble peptides in cheese and enzyme assays, pepN and pepX on the strains.

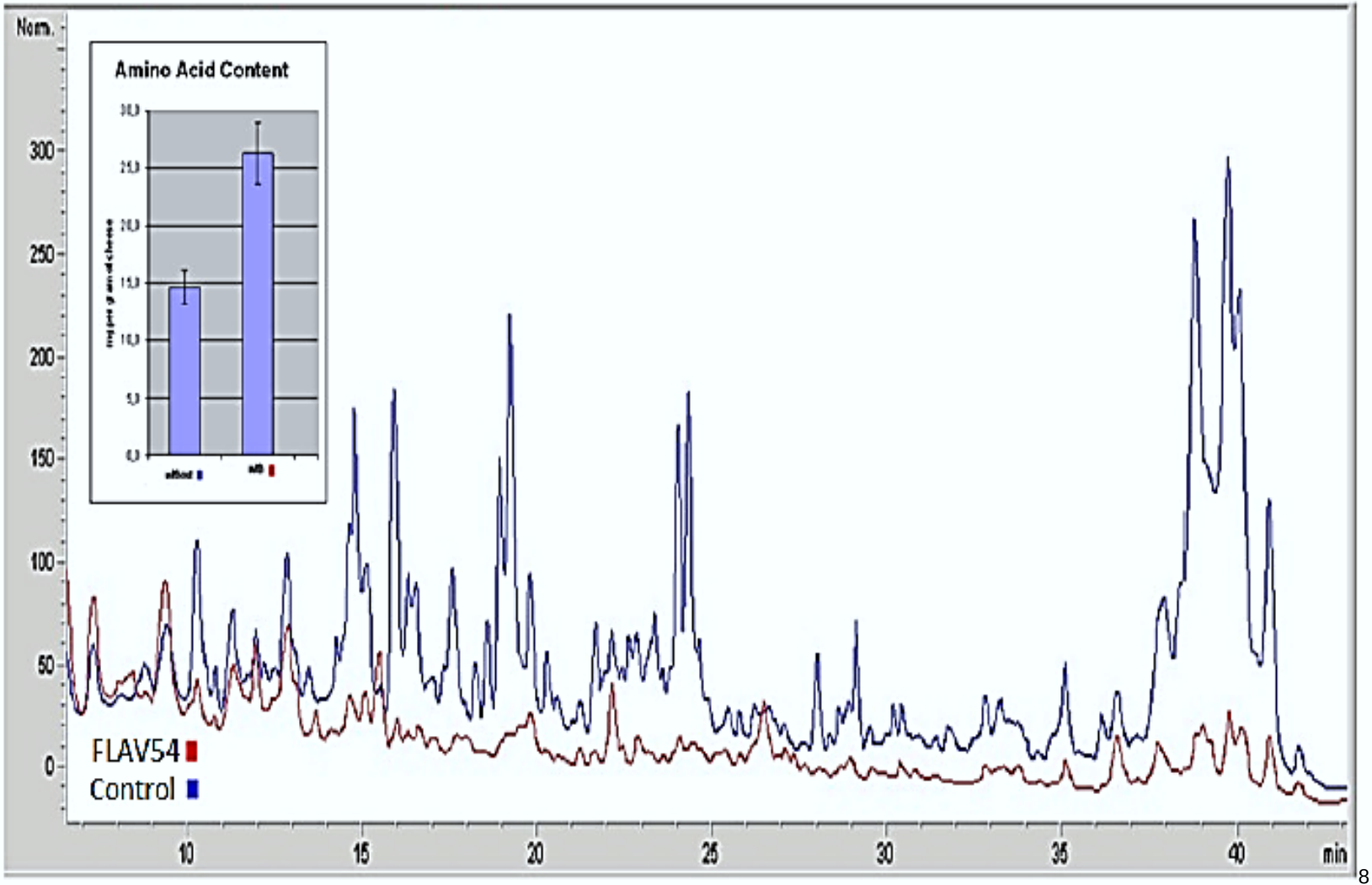


Free Amino Acid concentration

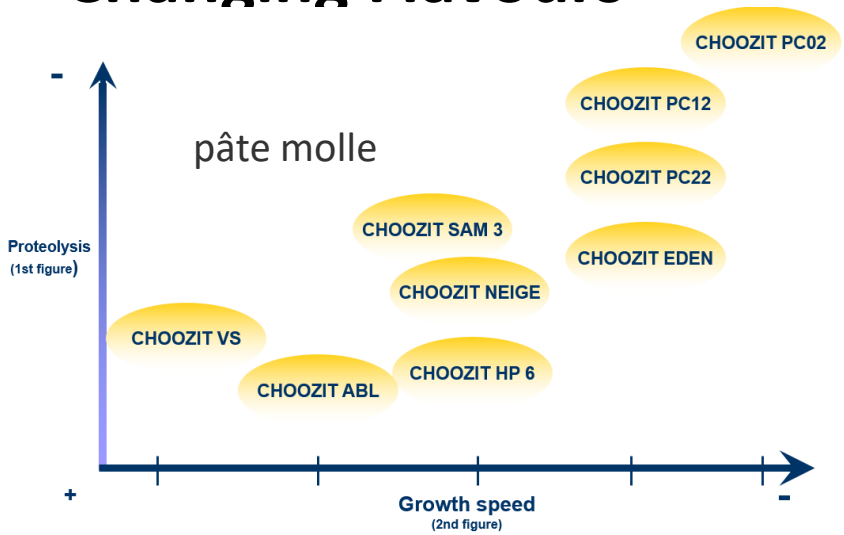
Measured by TNBS assay in cheese samples.
Absence of Amine Production in optimised media.

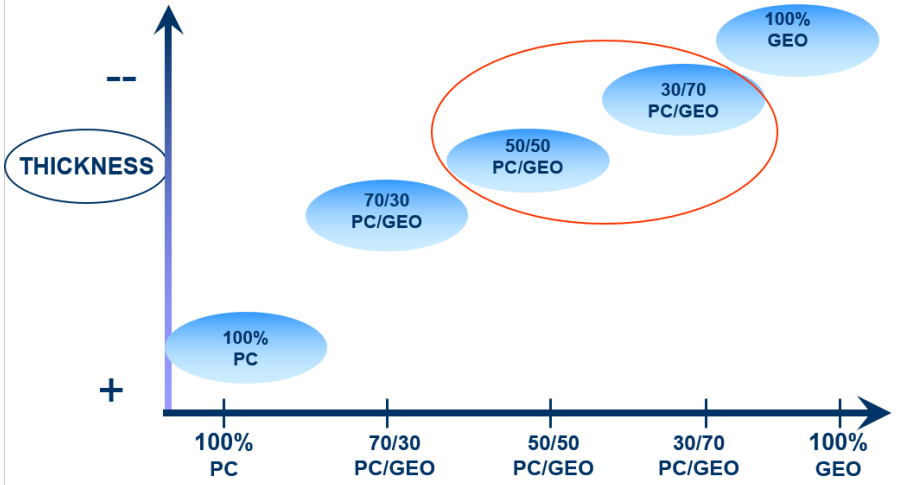


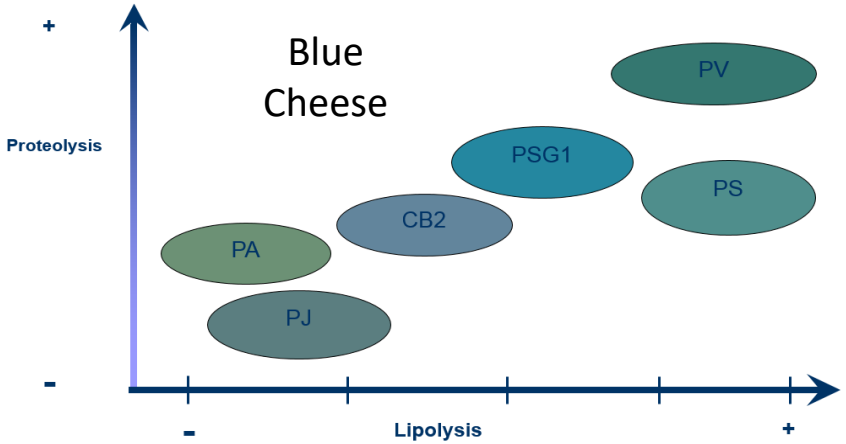
Impact of NSLAB cultures

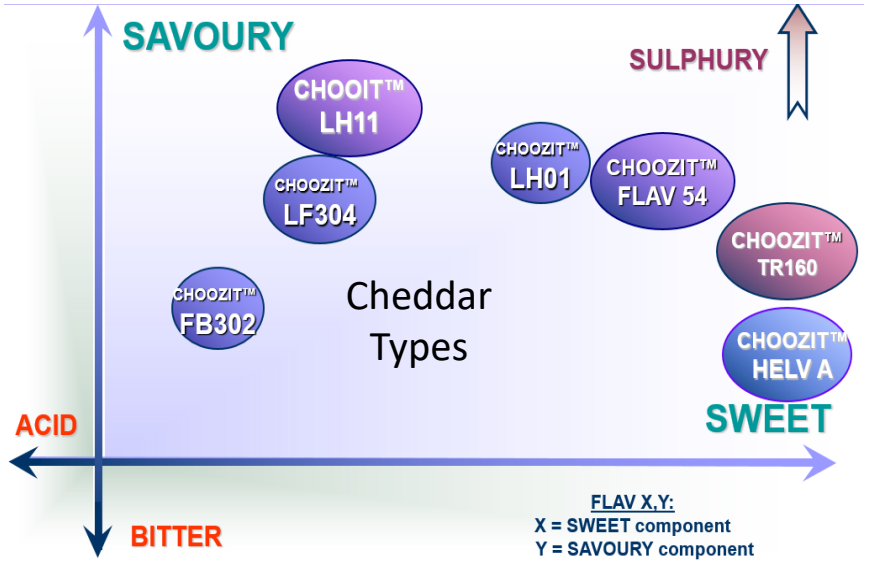


Changing Flavours





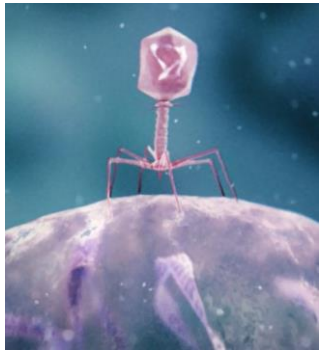




Starter cultures

- Crucial roles to play during all phases of the cheese making and maturation process.
- As the culture grows in the milk, it converts lactose to lactic acid. This ensures the correct pH for coagulation and influences the final composition of the cheese.
- The rate of acid production is critical in the manufacture of certain products, e.g. Cheddar cheese.
- In mechanized operations, starters are often required to produce acid at a consistently fast rate through the manufacturing period each and every day.
- Biggest threat to satisfactory performance is:

Bacteriophage



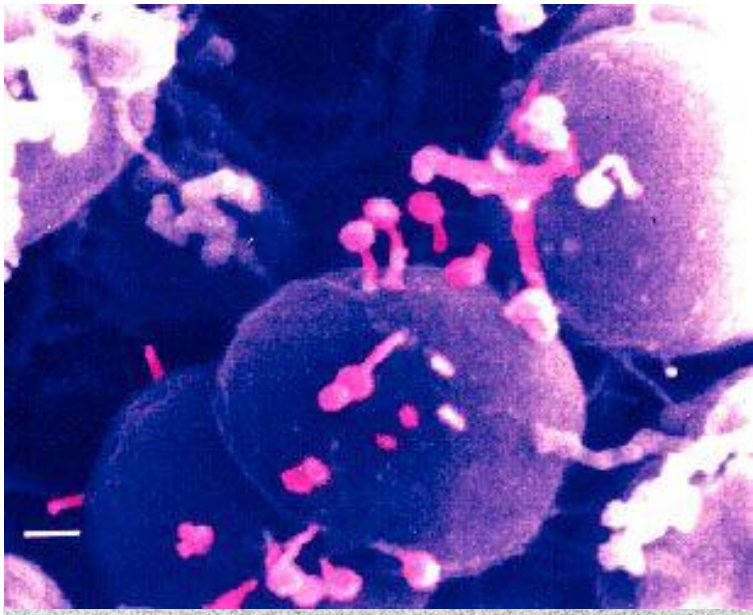
Phage Description

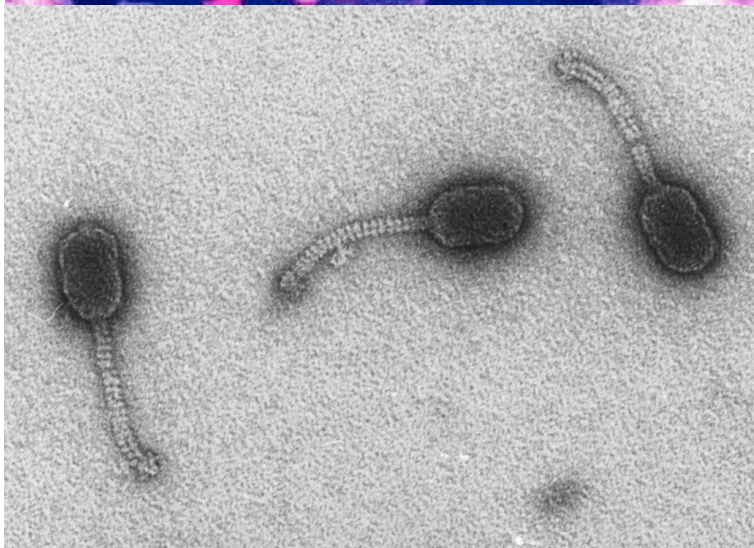
Phages are bacterial parasites (viruses), they requires bacteria to multiply.

Phages are very small living entities (10 times smaller than bacteria).

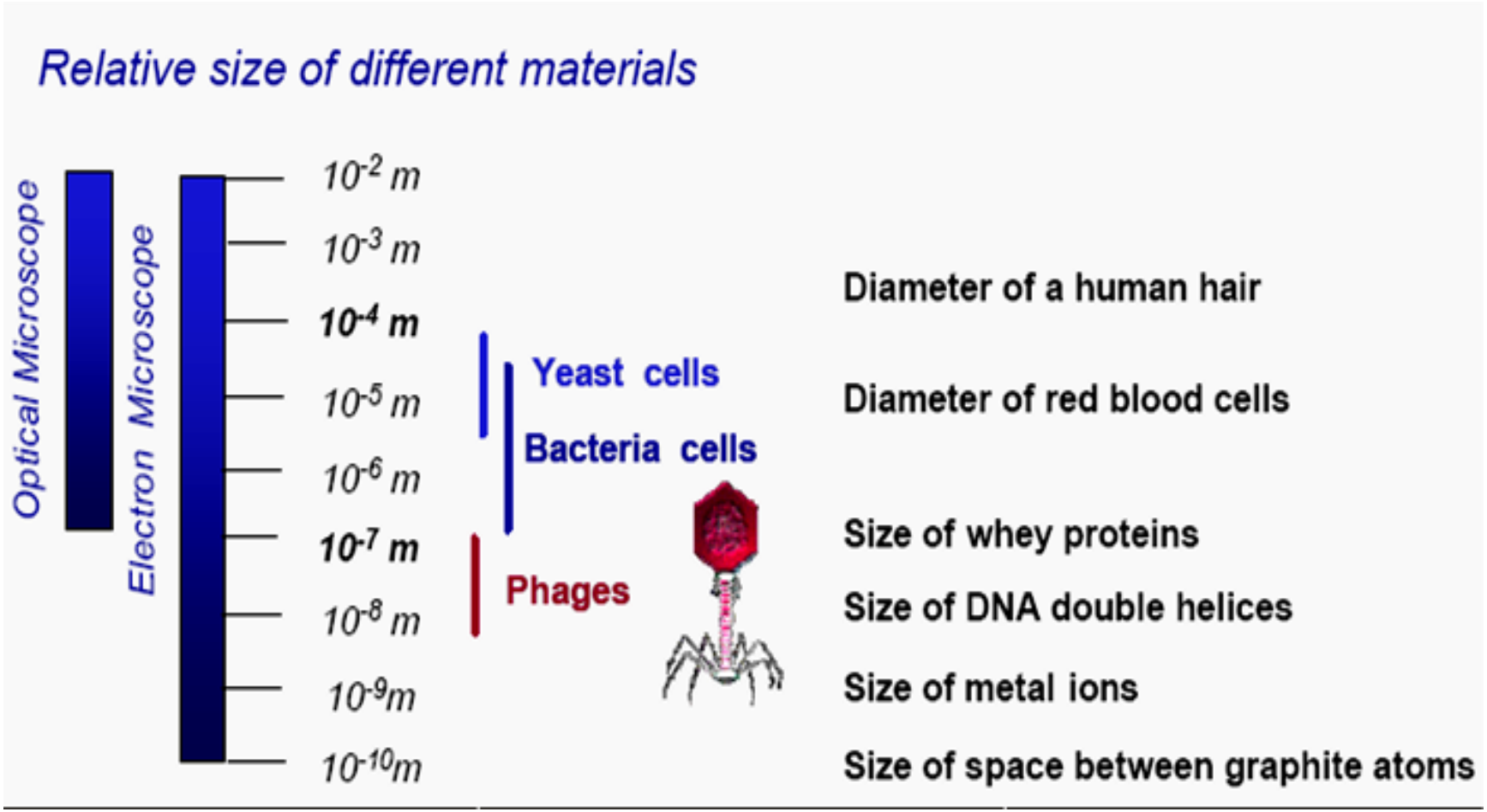
Phages are mainly made of proteins (head and tail). The phage head contains genetic material.

Their structure makes them very robust and difficult to eliminate



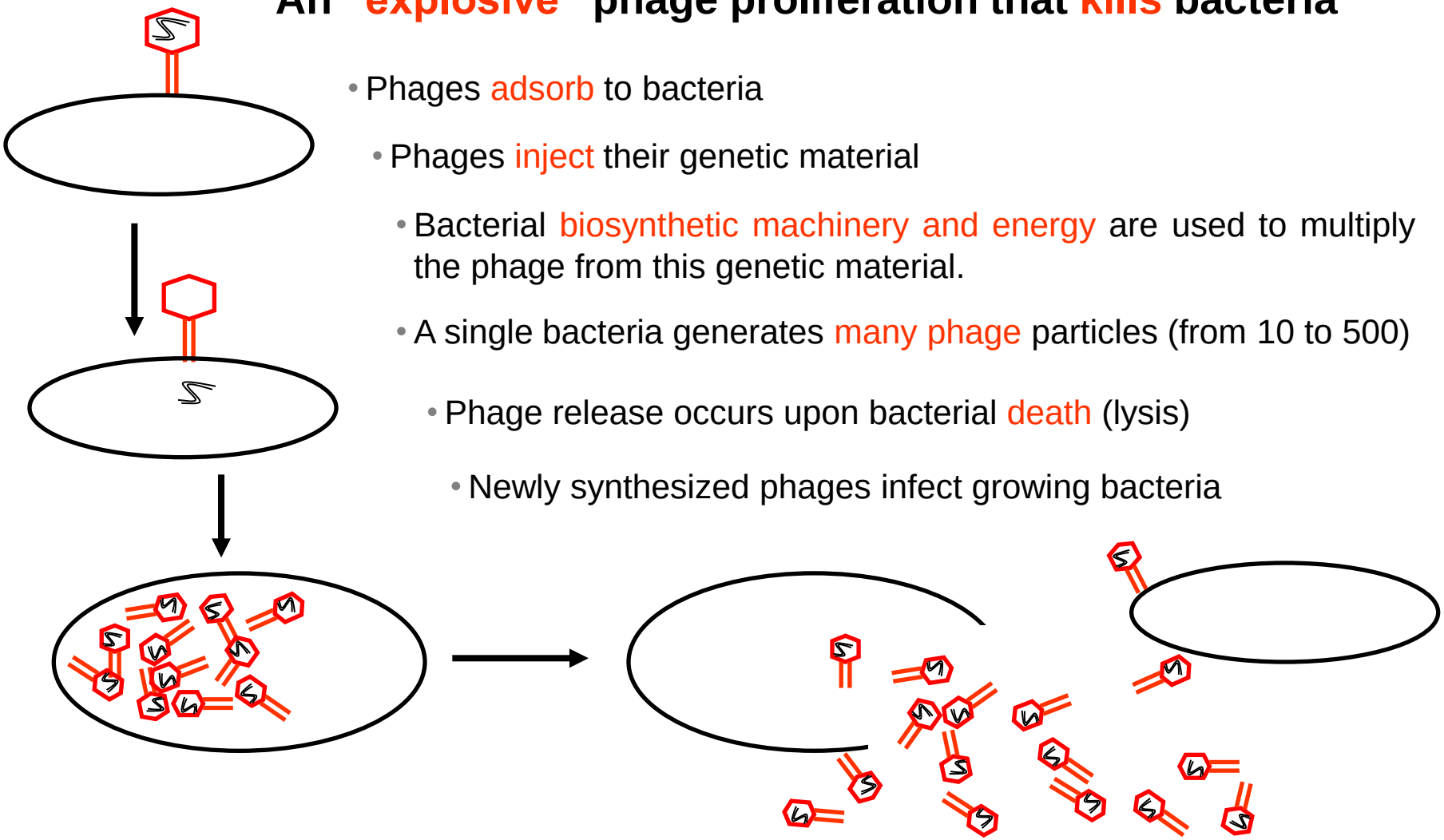


Bacteriophage are very small and omnipresent



Whitehead and Cox first demonstrated the role of phage in the failure of dairy fermentations in 1935

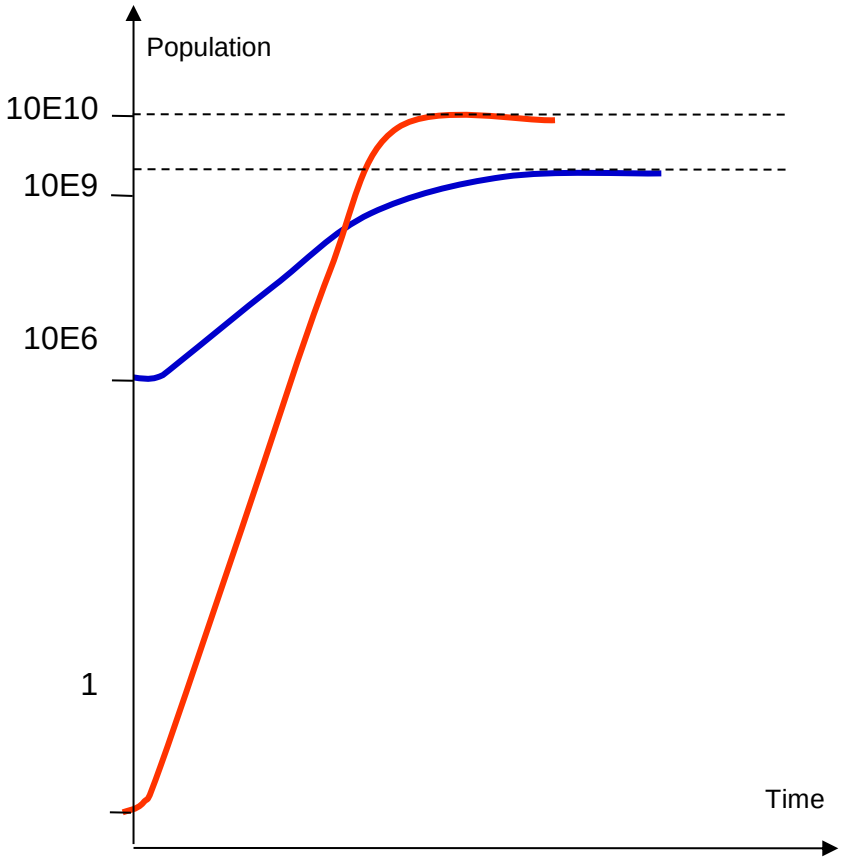
The Phage Biology : Phage Propagation



An “explosive” phage proliferation that kills bacteria

- Phages adsorb to bacteria
- Phages inject their genetic material
- Bacterial biosynthetic machinery and energy are used to multiply the phage from this genetic material.
- A single bacteria generates many phage particles (from 10 to 500)
- Phage release occurs upon bacterial death (lysis)
- Newly synthesized phages infect growing bacteria

The Phage Biology : Rate of Propagation



Time	Bacteria	Phages
30min	$1\ 10^6$	1
1h	$2\ 10^6$	$5.\ 10^1$
1h 30min	$4\ 10^6$	$2.5\ 10^3$
2h	$8\ 10^6$	$1.2\ 10^5$
2h 30min	$1.6\ 10^7$	$6.2\ 10^6$
3h	$3.2\ 10^7$	$3.1\ 10^8$
3h 30min	$6.4\ 10^7$	$1.6\ 10^{10}$
4h	$1.3\ 10^8$	
4h 30min	$2.6\ 10^8$	
5h	$5.2\ 10^8$	
5h 30min	$1.4\ 10^9$	

Amount of bacterial cells multiplies by 2 at each generation time

Phages proliferates faster than bacteria (possibly 50–70 times faster)

2. Sources of Phages

Phages present in the environment where they encounter bacteria.

- In milk (low level since there is little bacterial growth)
- In tankers (very low level if only milk is transported, higher risk if tankers are used for whey)
- In tanks and pipes (especially those containing fermented products)
- In atmosphere (spray)
- In plants (floor, wall (concrete), soil ...)
- In not well maintained CIP (make sure to maintain appropriate level of virucide products)
- In fermentation by-products (whey powders, whey cream...)

The ‘Phage Biology

Development of expertise on bacteriophage to study the lysotype of strains

Phages are collected from dairy plants world wide over years (about 7000 up to date)

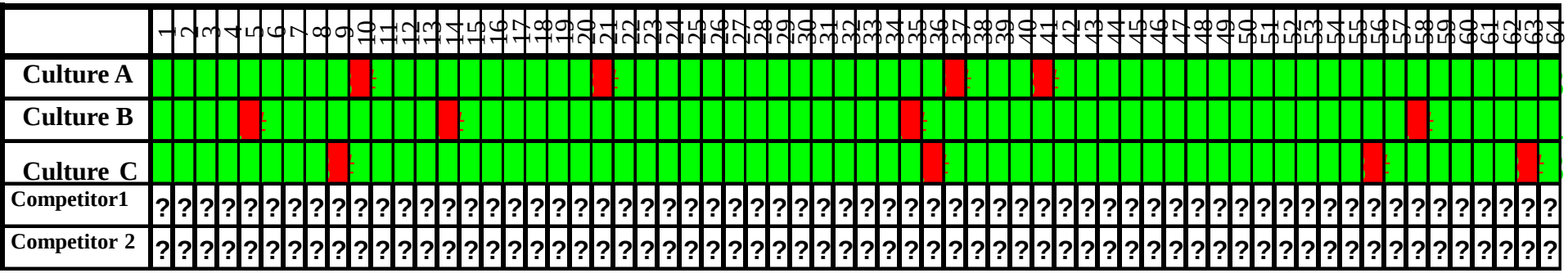
- analyzed to determine their biodiversity (host spectrum and genetic analyses) and classified
- analyzed to determine their potential technological impact (virulence, heat résistance)
- used to determine the lysotype of bacteria
- used to select new bacterial strains for the formulation of new starters
- Development of single colony isolates
  - Strains of known biological relationships
- Construction of culture rotations to minimize risk of attack

3. Fighting phages : Starter Rotation

The starters need to have different lysotype when used in rotation.

Using its collection of phages, Danisco determines the lysotype of each of its starters to make sure that they are appropriate rotation

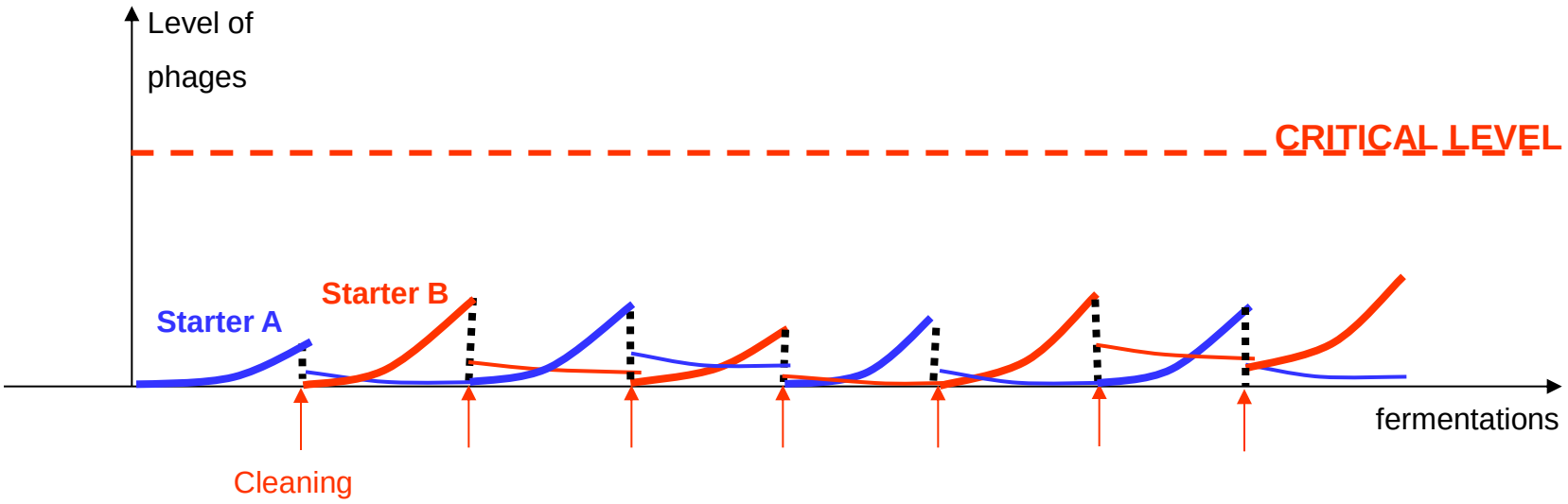
Example :



Bacteriophage evolves quickly. There is a constant need for vigilance

Fighting phage : Starter Rotation

The use in rotation of 2 or more starters helps to stabilise the level of phages in a dairy plant.



Starter rotation keep phage for a longer time below the critical level

Bacteriophage Management Strategy

- Limit starter culture propagation
- Appropriate use – dosage & rotation
- Sanitation of equipment
- Viricidal disinfection
- Continuous selection of new strains resistant to phage
- Renewal of starter cultures
- Rotation of starter cultures

Disadvantages of resistance

- Selected strains are not 100% identical
- Therefore starter cultures differ after their renewal
- Rotations are not absolutely isofunctional

The Swiss Knife approach

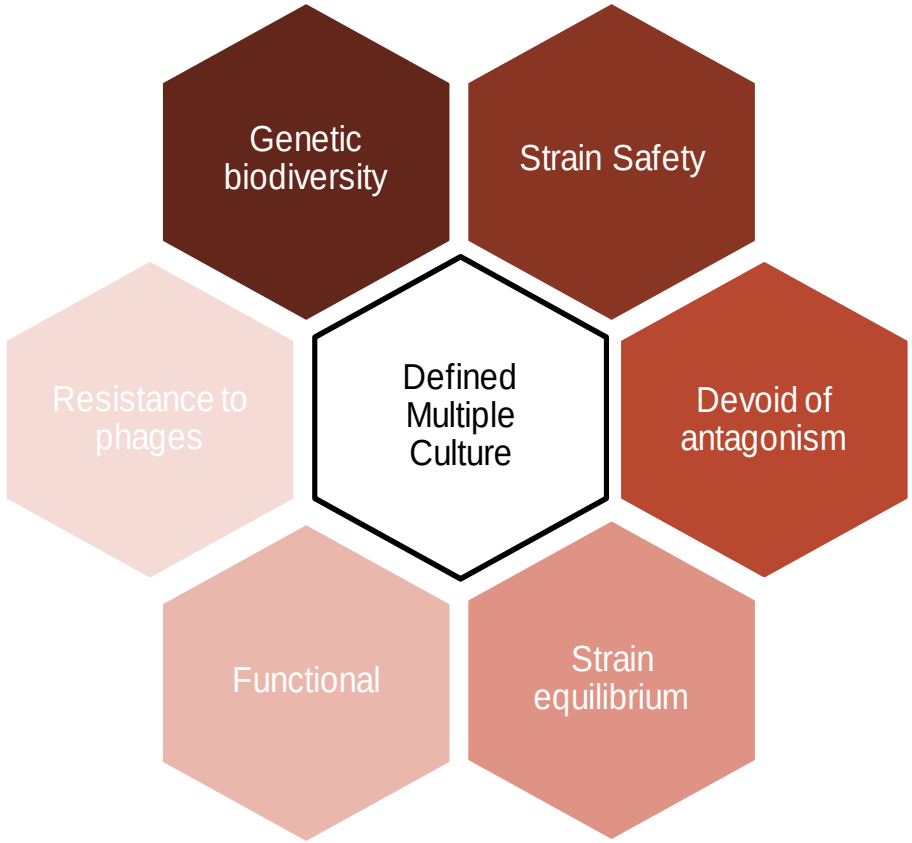
SIMPLE

COMPLEX

SELECTED MULTIPLE TOOLS

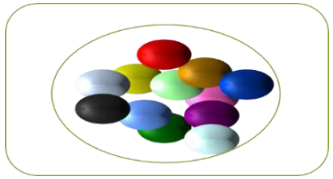
Defined multiple starter cultures
The science behind the formulation

- **Strain selection process**
  - 64 strains
- **Starter culture development**
  - More than 10 stains per starter
- **Genetic biodiversity**
- Genome sequencing for each strain
- To make sure strains are distinct
- To develop methods for the specific detection of each strains
- **Devoid of antagonism**
- Ability to produce antagonistic molecules
- One to one strain testing
- Antagonistic strains discarded



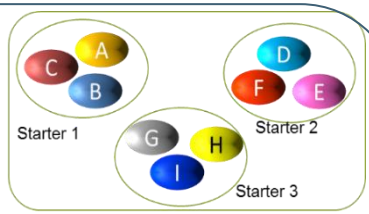
The Swiss Knife approach

COMPLEX STARTERS CULTURES



- Interesting biodiversity but difficult to control
- Intensive industrial efforts to achieve a cheese process consistency
- The phage alternative concept doesn't exist

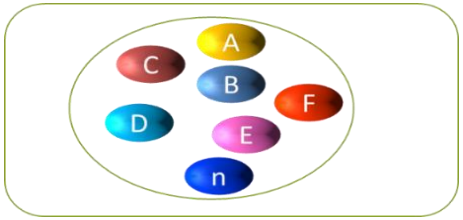
DEFINED STARTERS CULTURES



- Exact definition of the target starter properties
- High cheese process consistency
- Phage rotation program defined with the customer

A new formulation approach : DEFINED MULTIPLE STARTER CULTURES

- Starting with selection of the target cultures properties
- Rotation concept is possible and has been developed
- No trade off on starter cultures properties
- Facilitate starters cultures performance and consistency

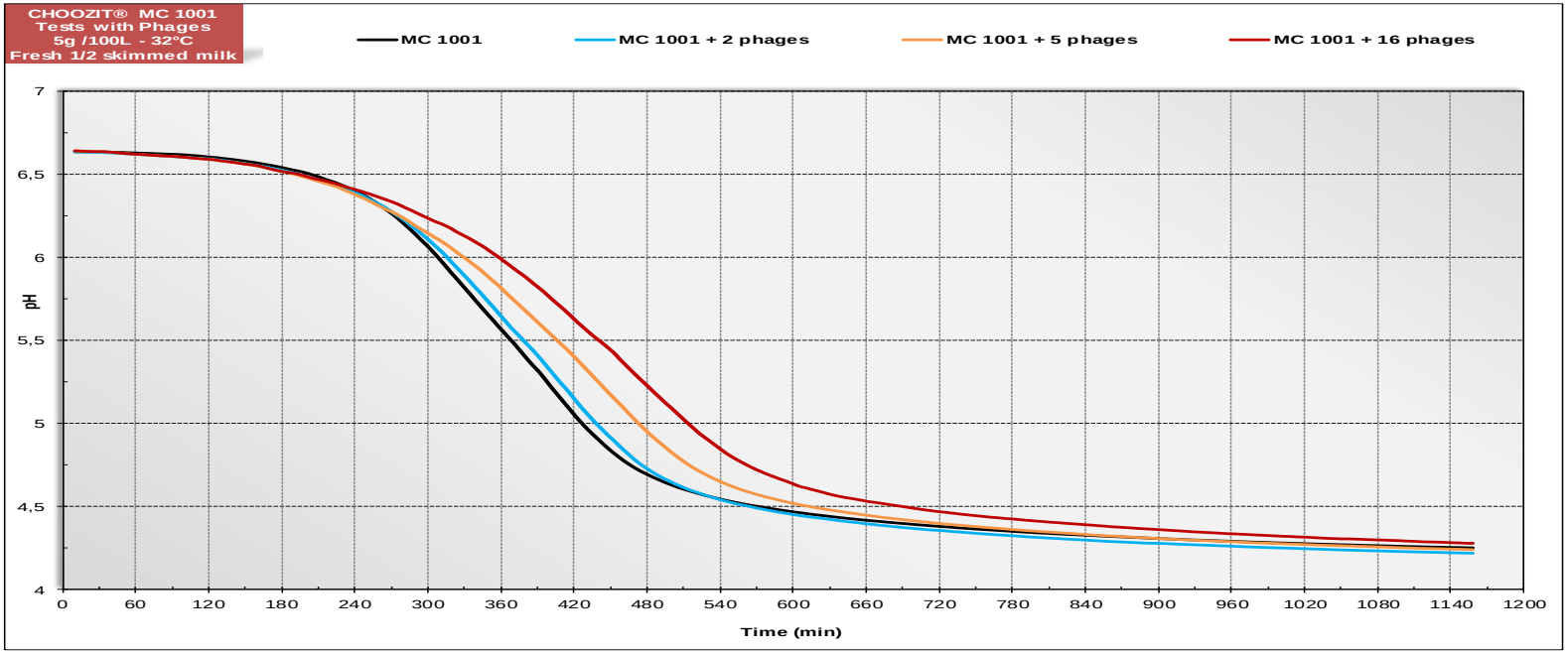


CHOOZIT® 1000 series

Defined multiple starter cultures
The science behind the formulation

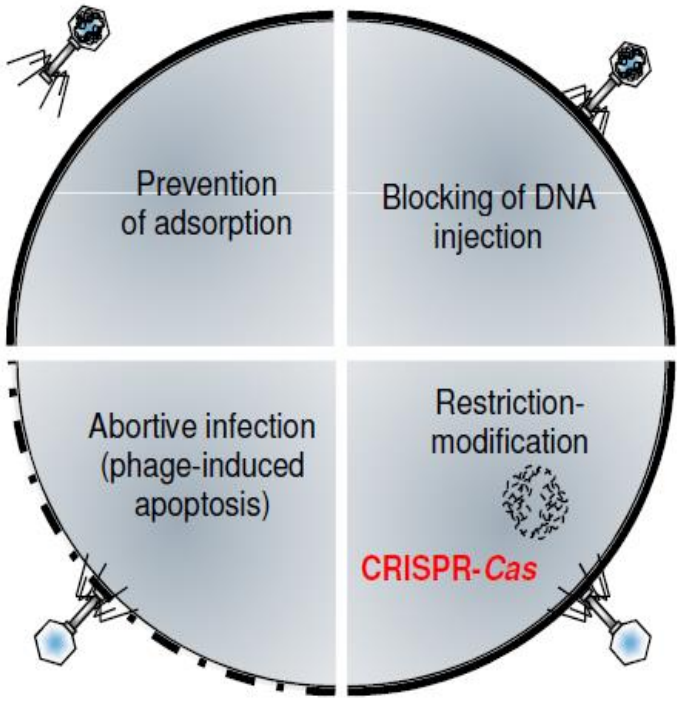
- **Strain equilibrium**
  - Upon production process
  - Risk to have some strains dominating
  - Analysis of strain ratio upon production
  - Thanks to genomics data and the development of a q-PCR method

- **Resistance to phages**
  - Thanks to biodiversity, little impact of reasonable phage attack
  - No dead-vat upon massive phage attack on the contrary to simple starter cultures

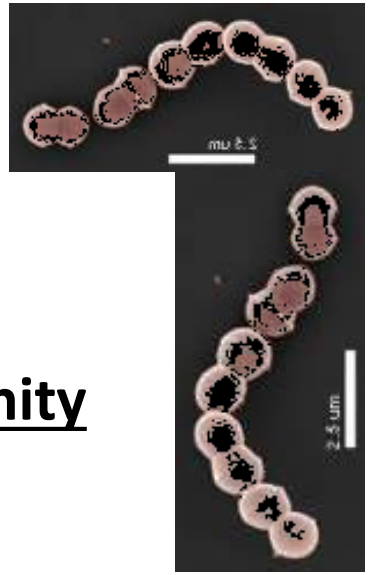


Bacteriophage resistance mechanisms

- To resist bacteriophage attack strains have developed many different strategies
- Among the classic strategies are:
  - Prevention of absorption
  - Blocking injection
  - Abortive infection
  - Restriction/modification
  - ...etc
- Recently, DuPont scientists have put in evidence a new bacteriophage resistance mechanism based on the destruction of the bacteriophage DNA (restriction)
  - CRISPR-*Cas* system



CRISPR technology

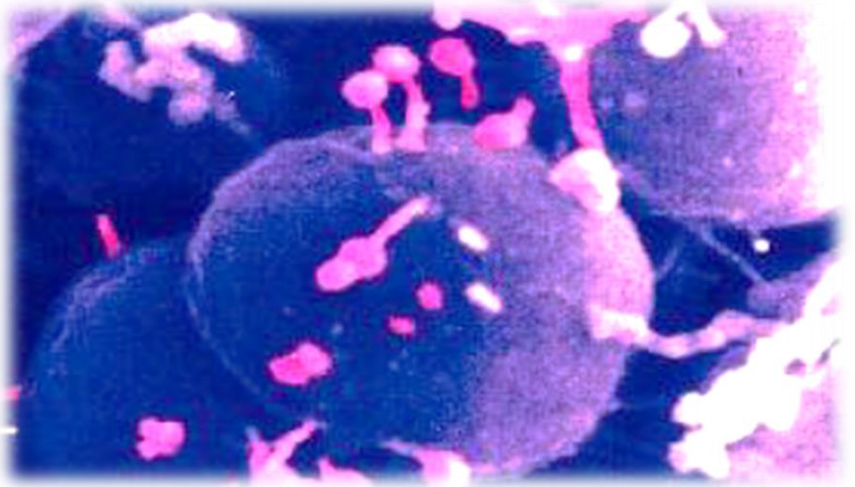


Danisco uncovers mechanism behind bacterial immunity

Important findings from joint research project published in Nature

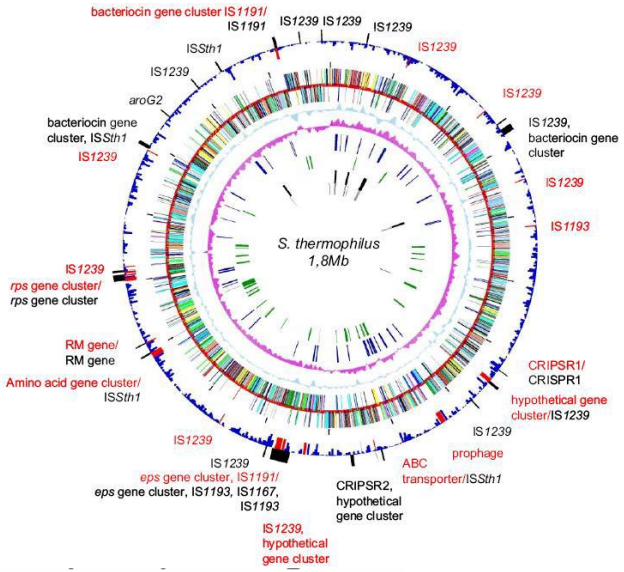
Joint research conducted between Danisco and Universitè Laval has shed light on the mechanisms behind the natural bacterial immune system CRISPR. The findings highlight the valuable potential as a simple natural way to generate more robust organisms with built in resistance to virus attacks. CRISPR research has generated results that can be used to generate more robust Starter cultures by improving the natural Defence systems in

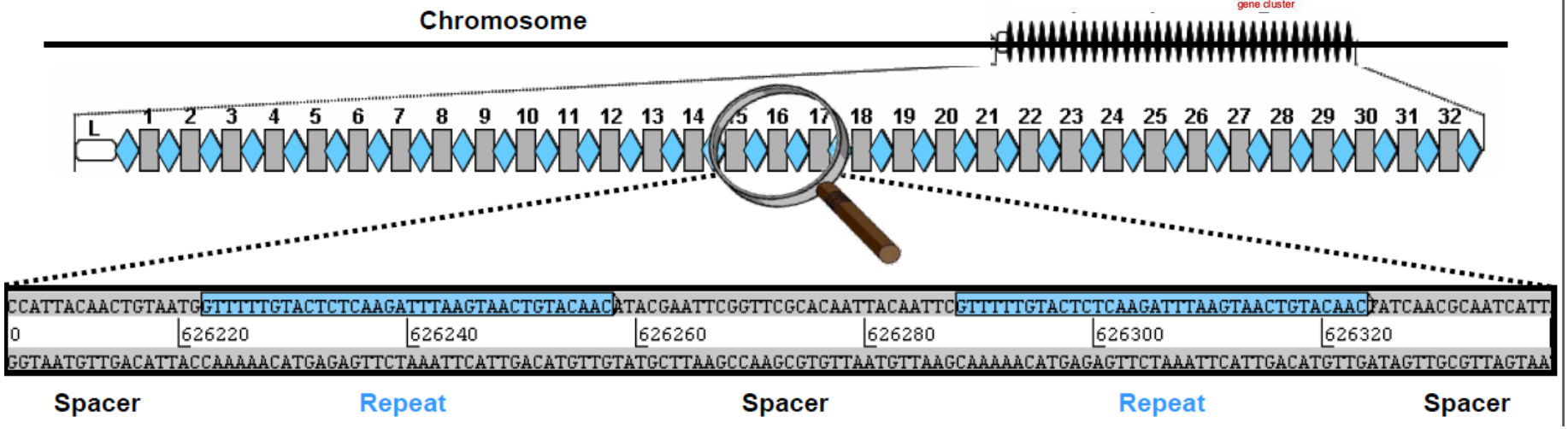
Streptococcus thermophilus



CRISPR Resistance to Bacteriophages

- CRISPR is an acronym for
  - **C**lustered
  - **R**egularly
  - **I**nterspaced
  - **S**hort
  - **P**alindromic
  - **R**epeats





Bacteriophage management - CRISPR

Immunization of cultures: Securing unchanged performance

- The CRISPR system is more than a classic bacteriophage resistance mechanism
- The CRISPR system acts as a restriction system by destroying bacteriophage DNA upon infection
- It is an *evolutive* resistance mechanism; an *immunization system*
- Upon encountering bacteriophage the CRISPR system acquires additional resistance capabilities
- Each bacteriophage encounter allows the acquisition of additional resistances
- *Evolution of CRISPR system occurs spontaneously and naturally upon bacteriophage infection*
- Acquired bacteriophage resistances accumulate in a strain and are inheritable

CRISPR Application to pizza cheese

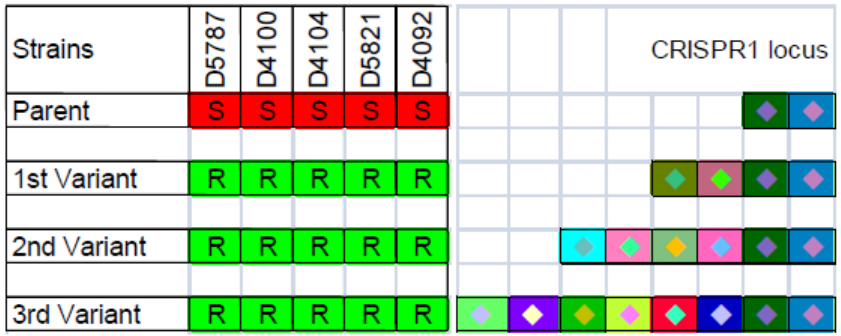
Step 1: Definition of a series of representative phage

- 17 phage for strain C and 37 phage for strain D

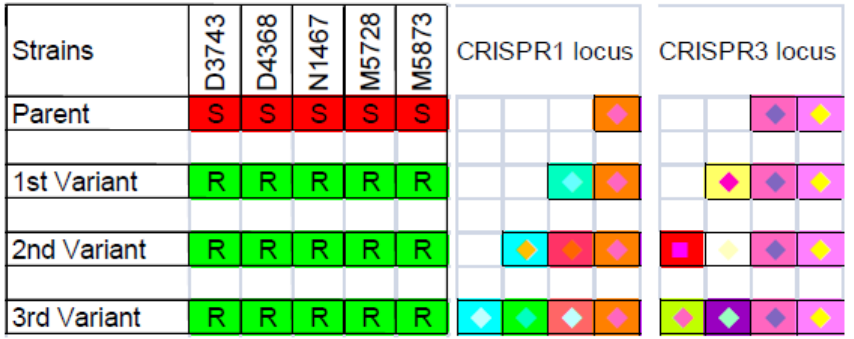
Step 2: Selection of 3 CRISPR variants resistant to phage for each strain

- 3 independent variants for each strain each presenting different new spacers in their CRISPR loci
- All variants are totally resistant to the panel a representative phage

Strain C



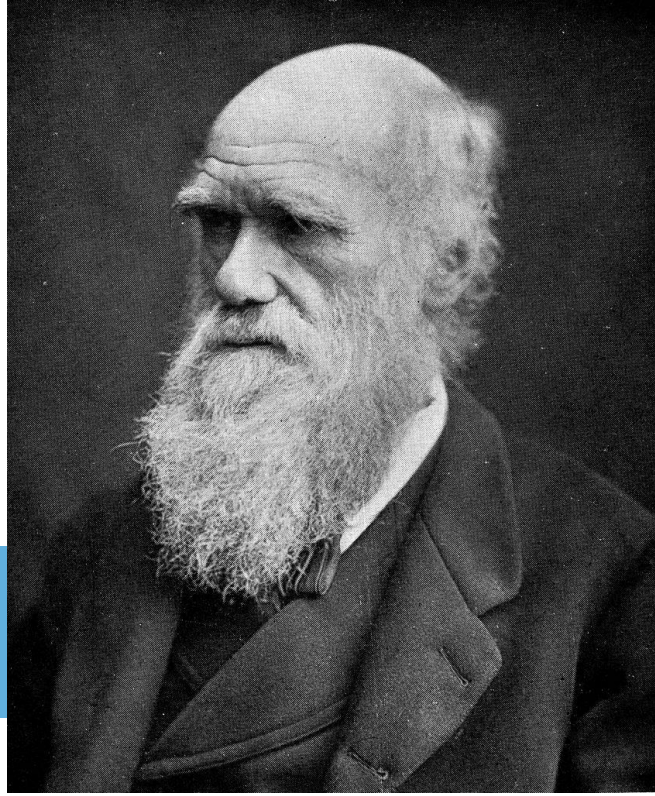
Strain D



THANK YOU

DUPONT®

DANISCO®



I have called this principle, by which each slight variation, if useful, is preserved, by the term of Natural Selection.