Istituto Zooprofilattico Sperimentale della Sicilia
"A.Mirri"

Il Microbioma dei formaggi tipici Siciliani in relazione ai disciplinari di produzione. Esempio di metagenomica applicata

Produzioni casearie tipiche siciliane

Formaggi analizzati e loro caratteristiche

Pasta Filata

Pasta Dura

Prodotti con latte di pecora crudo in tini di legno

40° C

Formaggi analizzati e loro caratteristiche

Formaggi tradizionali

Ampia varietà di caratteristiche sensoriali

- Caratteristiche biochimiche del latte
- Fattori ambientali
- Processi di lavorazione
- Razza animale
- L'evoluzione e il metabolismo dei batteri

Formaggi e batteri

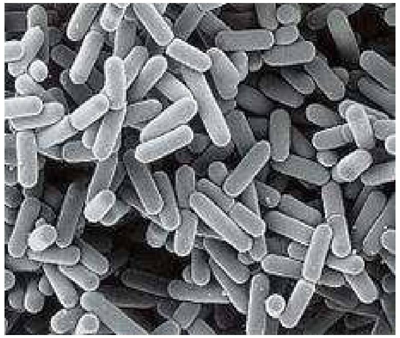
Batterici lattici (LAB)

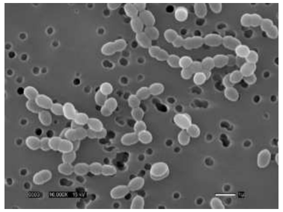
Lactococcus lactis subsp. *lactis*
Lactococcus lactis subsp. *diacetilactis*
Lactococcus lactis subsp. *cremoris*
Leuconostoc mesenteroides subsp. *cremoris*
Leuconostoc lactis
Lactobacillus acidophilus
Lactobacillus casei subsp. *casei*
Lactobacillus casei subsp. *rhamnosus*
Lactobacillus casei subsp. *pseudoplantarum*

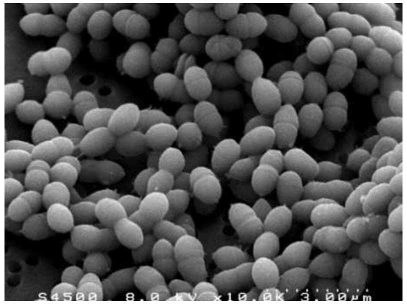
BATTERI
LATTICI
MESOFILI

Streptococcus thermophilus
Lactobacillus delbrueckii subsp. *bulgaricus*
Lactobacillus delbrueckii subsp. *lactis*
Lactobacillus helveticus
Pediococcus acidilactici

BATTERI
LATTICI
TERMOFILI



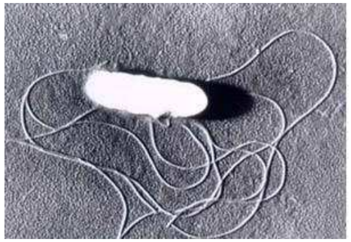


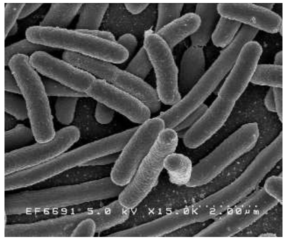


Formaggi e batteri

Batterici patogeni potenzialmente presenti

- *Staphylococcus aureus*
- *Listeria monocitogenes*
- *Salmonella spp.*
- *Escherichia coli*
- *Campylobacter spp.*



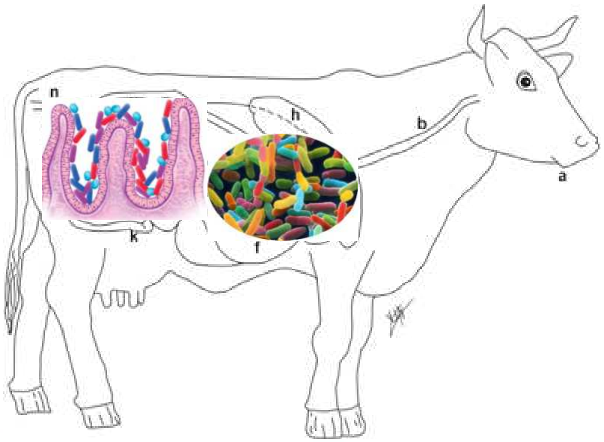


Importanza dei sistemi di controllo

Sicurezza

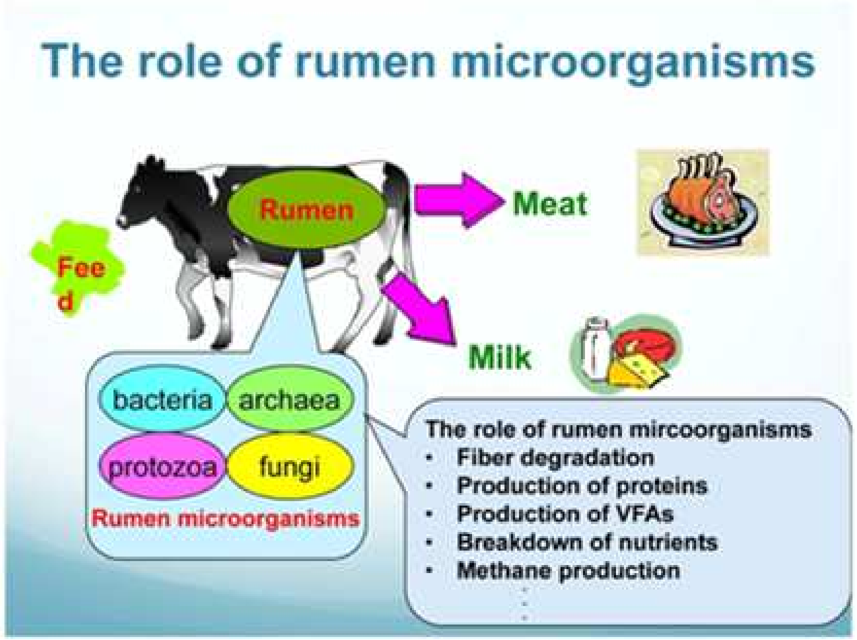
Qualità

Microbiota Animale



influenza

- Lo stato di salute
- Il benessere
- **La fisiologia**
- **pH ruminale**
- **L'accrescimento**
- La composizione del latte (tenore in grasso, in lattosio e proteine)
- **la qualità della carne**


The role of rumen microorganisms

The role of rumen mircoorganisms
- Fiber degradation
- Production of proteins
- Production of VFAs
- Breakdown of nutrients
- Methane production

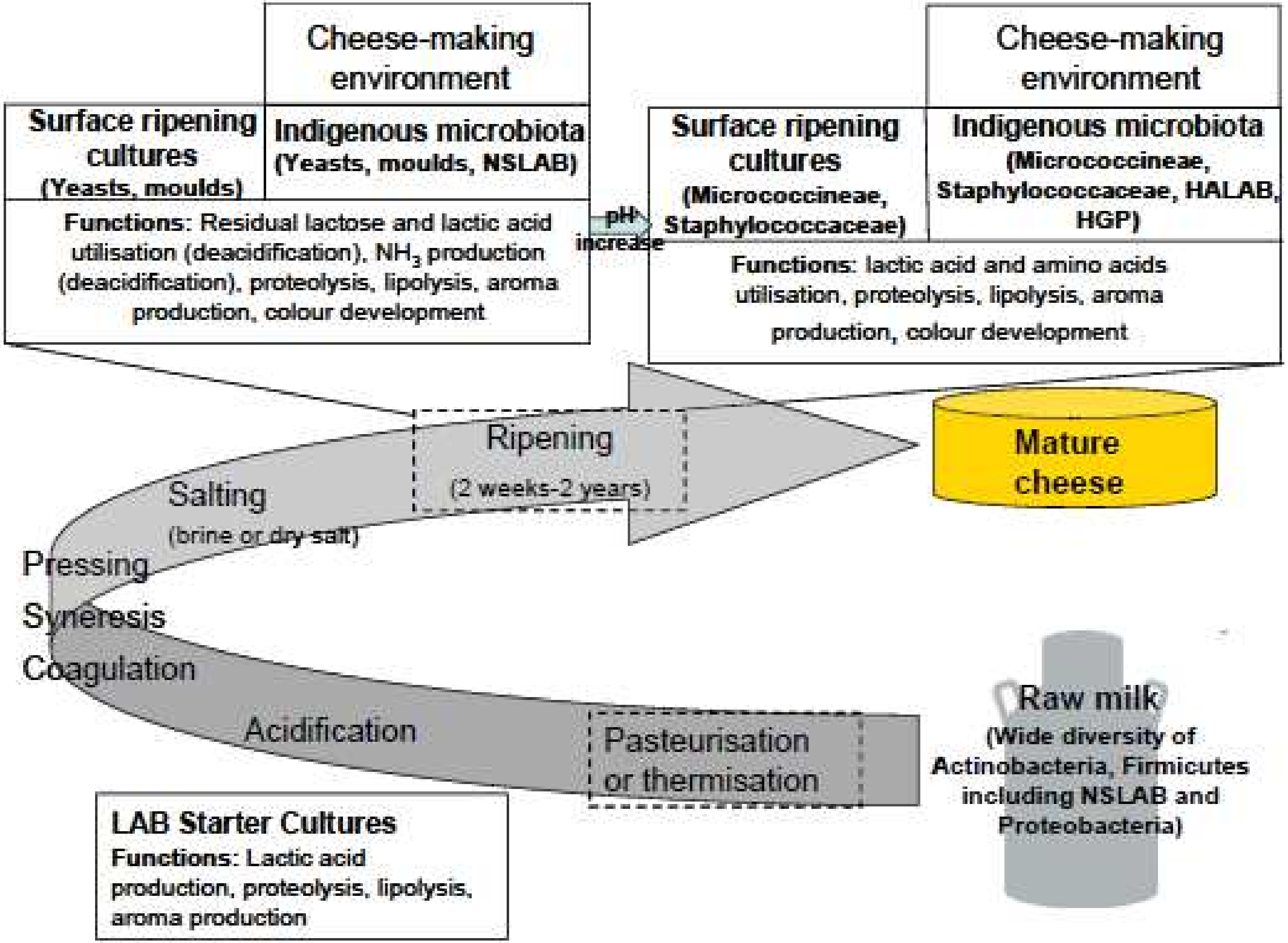

Cheese-making environment
Surface ripening cultures (Yeasts, moulds)
Indigenous microbiota (Yeasts, moulds, NSLAB)
Functions: Residual lactose and lactic acid utilisation (deacidification), NH_3 production (deacidification), proteolysis, lipolysis, aroma production, colour development
pH increase
Cheese-making environment
Surface ripening cultures (Micrococcineae, Staphylococcaceae)
Indigenous microbiota (Micrococcineae, Staphylococcaceae, HALAB, HGP)
Functions: lactic acid and amino acids utilisation, proteolysis, lipolysis, aroma production, colour development
Ripening
(2 weeks-2 years)
Mature cheese
Salting
(brine or dry salt)
Pressing
Syneresis
Coagulation
Acidification
Pasteurisation or thermisation
Raw milk
(Wide diversity of Actinobacteria, Firmicutes including NSLAB and Proteobacteria)
LAB Starter Cultures
Functions: Lactic acid production, proteolysis, lipolysis, aroma production

Table 1. Inventory of microbial interactions in cheeses

Interaction types	Effect in cheese and / or molecules produced	Microorganisms involved	Microbiological phenomenon observed
Commensalism	Cheese-surface deacidification: Lactic acid utilisation Alcaline metabolites (NH_3) production	Yeasts, ripening bacteria	Growth of acid-sensitive bacteria
Amensalism	Curd acidification Organic acids (i.e. lactate)	LAB, spoilage and pathogenic bacteria	Inhibition of acid-sensitive bacteria
	Bacteriocins		Lysis of pathogenic and spoilage bacteria
Competition	Harvest of Iron	Siderophores containing bacteria, auxotrophic bacteria	Reduced colonization capacity of auxotrophic strains
	Jameson effect	Microbiota of wooden shelves, *Listeria monocytogenes*	Limited colonization of *L. monocytogenes*
Parasitism	Failure of fermentation	Phage, bacteria	Inactivation of dominant strains

Abbreviations: LAB, lactic acid bacteria

Scopo del lavoro

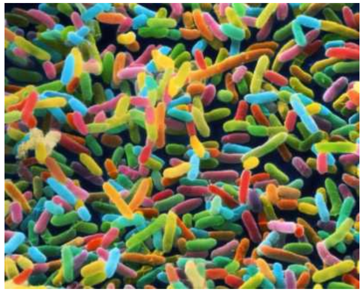
Identificazione della composizione batterica (microbioma) dei formaggi attraverso

Next Generation Sequencing (NGS)

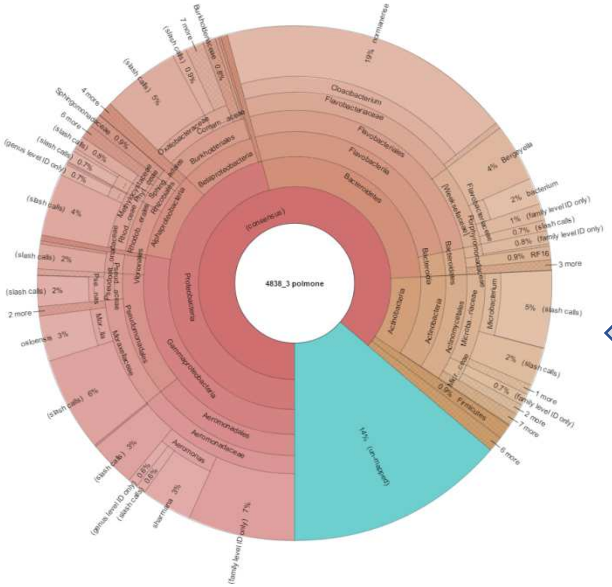
16S rRNA sequencing

Estrazione DNA

Sequenziamento



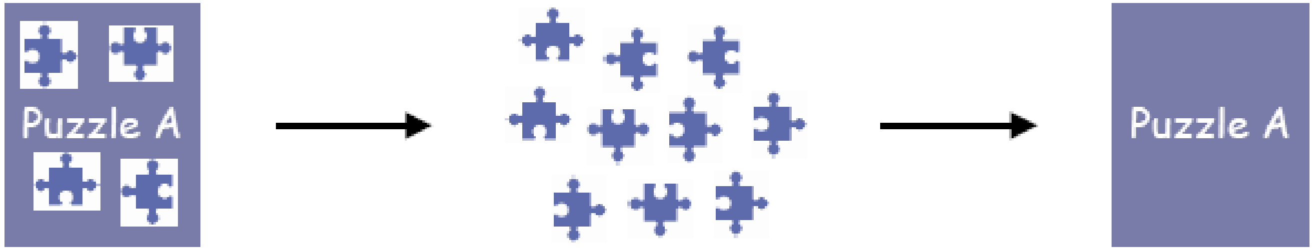
Analisi tassonomica



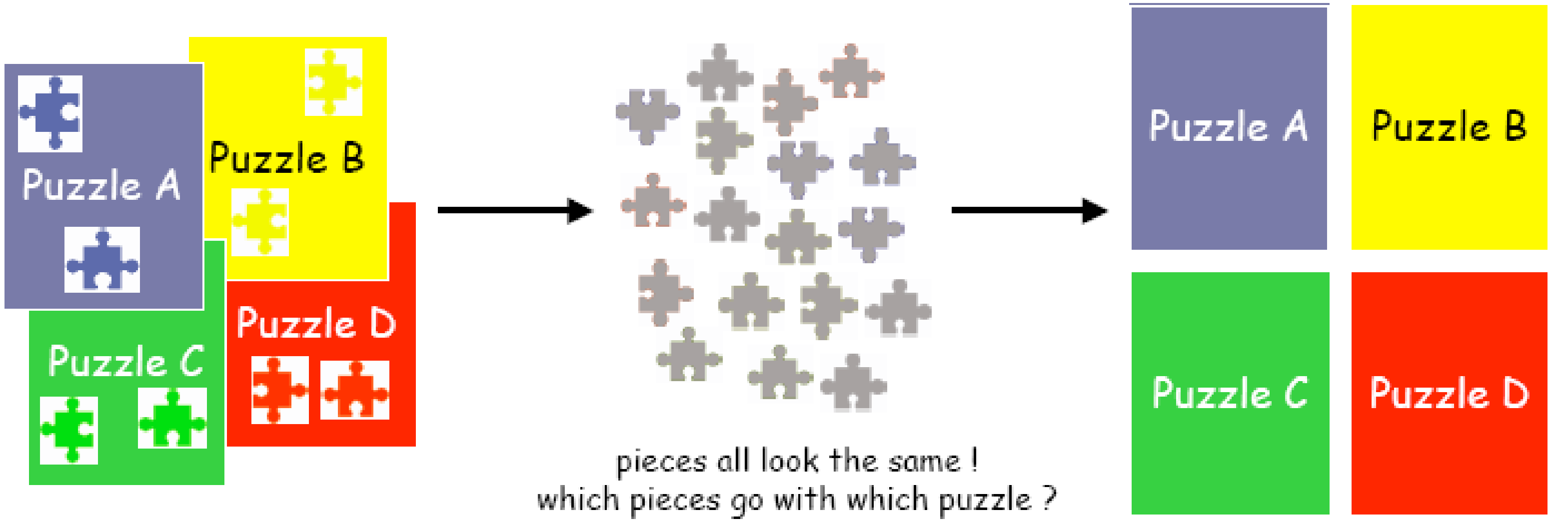
Caratterizzazione comunità microbica

Phylum – classe – ordine – famiglia – genere – specie

Isolate genome - single source of DNA



Environmental genome - multiple sources of DNA



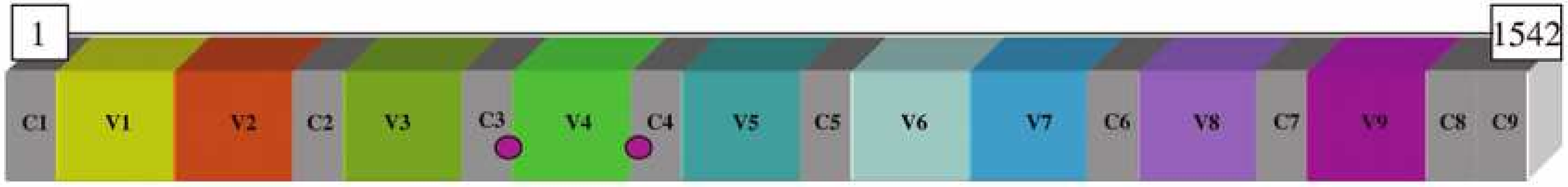
pieces all look the same !
which pieces go with which puzzle ?

Materiali

Numero Interno	Proprietà	Specie	Azienda
1	Tino castagno calabrese	Ovina	Izs Sicilia
2	Tino castagno madonita	Ovina	Izs Sicilia
3	Tino Cedro	Ovina	Izs Sicilia
4	Tino ciliegio	Ovina	Izs Sicilia
5	Tino frassino	Ovina	Izs Sicilia
6	Tino noce	Ovina	Izs Sicilia
7	Tino pino	Ovina	Izs Sicilia
8	Tino pioppo	Ovina	Izs Sicilia
9	Tino castagno calabrese	Ovina	Izs Sicilia
10	Tino castagno madonita	Ovina	Izs Sicilia
11	Tino Cedro	Ovina	Izs Sicilia
12	Tino ciliegio	Ovina	Izs Sicilia
13	Tino frassino	Ovina	Izs Sicilia
14	Tino noce	Ovina	Izs Sicilia
15	Tino pino	Ovina	Izs Sicilia
16	Tino pioppo	Ovina	Izs Sicilia
17	Vastedda Listeria	Ovina	Izs Sicilia
18	Pecorino Listeria	Ovina	Izs Sicilia

Metodi

1. Estrazione del DNA
2. Amplificazione del 16S rDNA



NGS

Costruzione di una libreria genomica

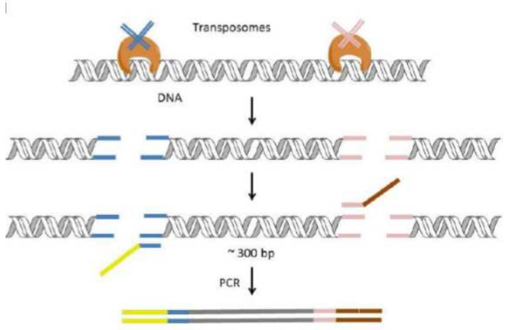
Frammentazione del DNA genomico 1

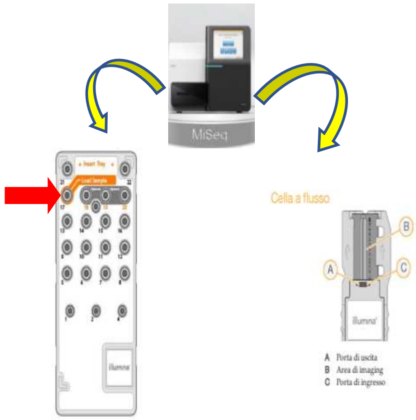
Amplificazione dei frammenti di DNA 2

Purificazione del DNA amplificato 3

Normalizzazione delle librerie 4

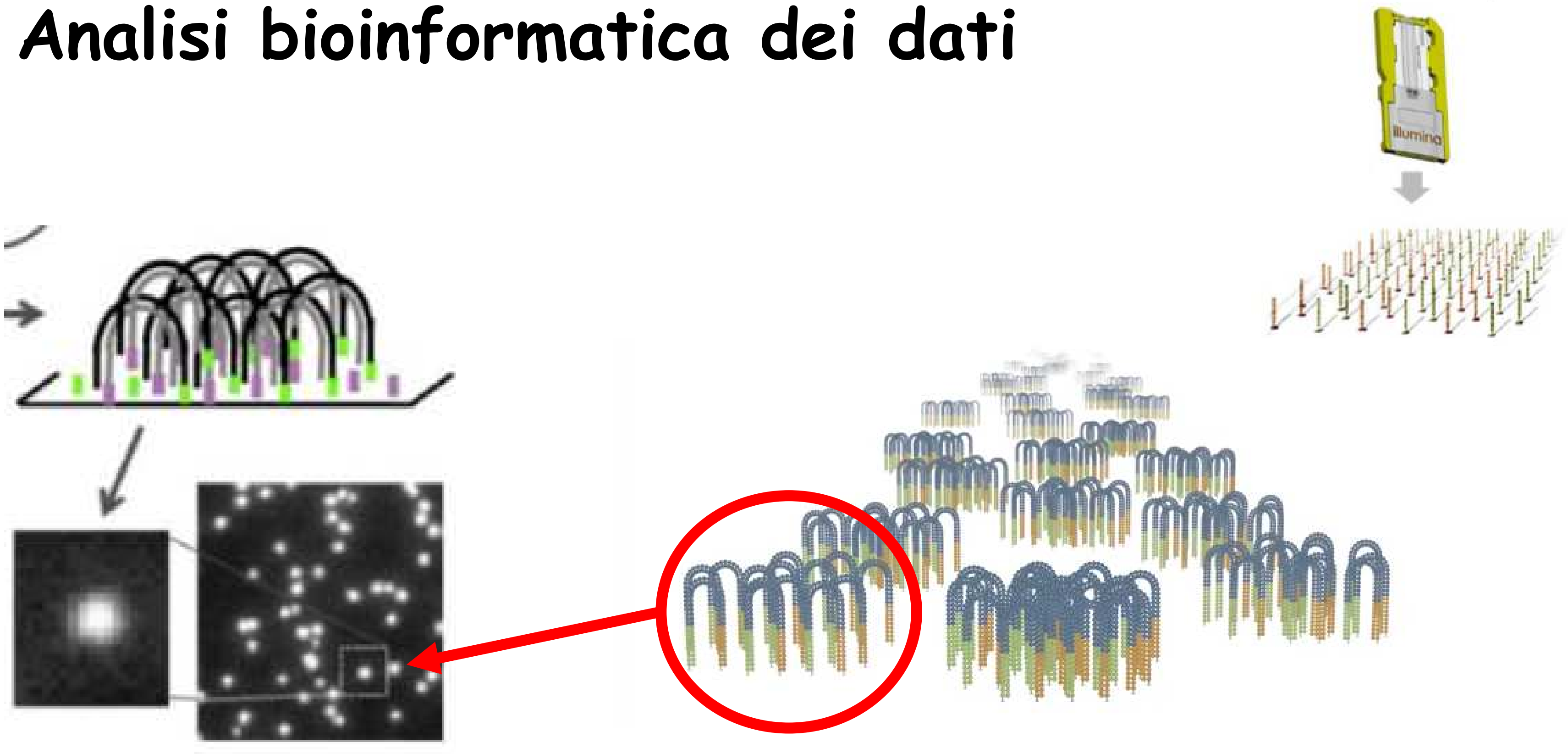
***Pool* delle librerie** 5





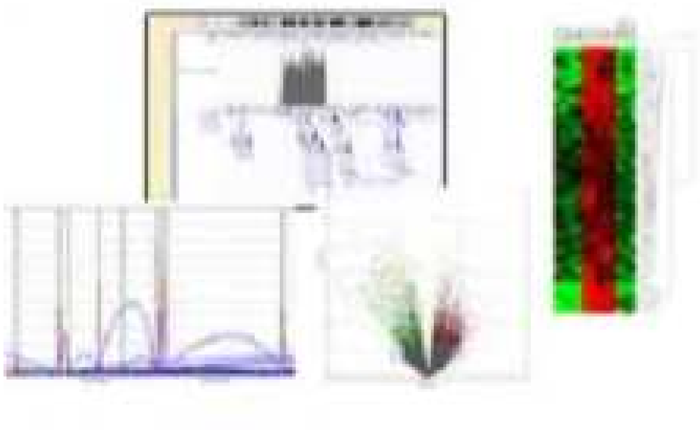
NGS

1. Amplificazione clonale della libreria
2. Sequenziamento ciclico e imaging
3. Analisi bioinformatica dei dati



NGS

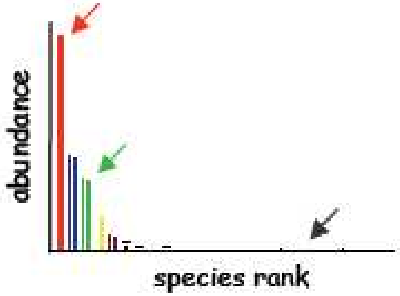
1. Amplificazione clonale della libreria
2. Sequenziamento ciclico e imaging
3. **Analisi bioinformatica dei dati**

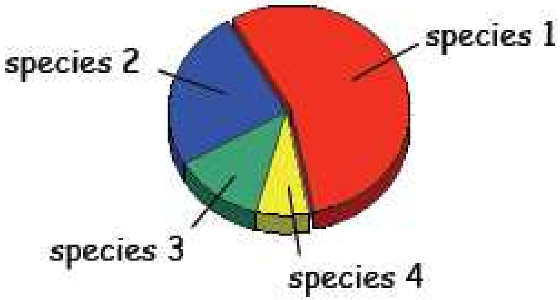


Software MiSeq Reporter (MSR)

Estimation of species richness and eveness

- 16S rRNA gene library analysis
- fluorescent in situ hybridization



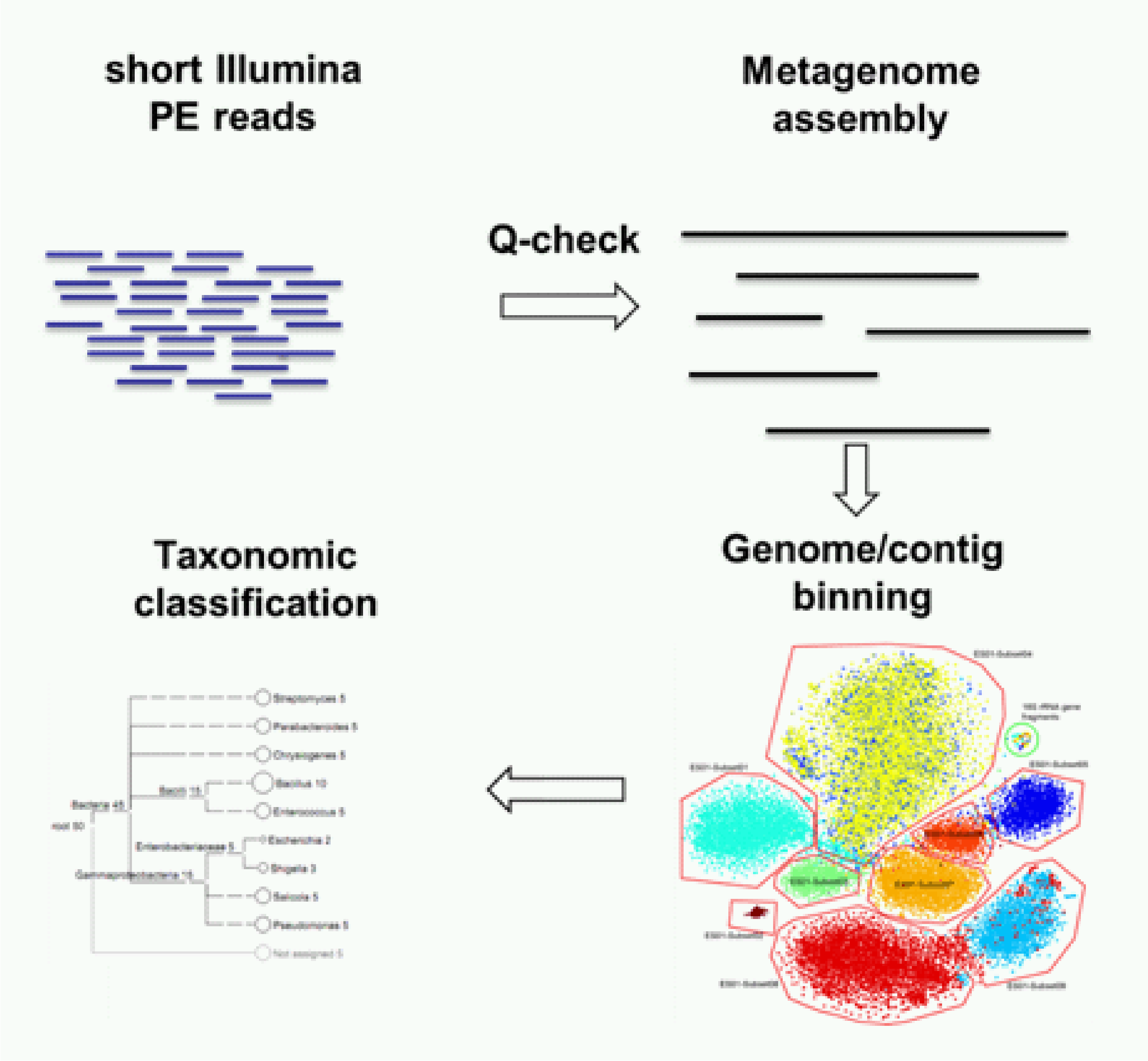


Genome **coverage** is proportional to **abundance** in the **library**

abundant types >> moderately abundant types > rare types

Table 2. Recent applications of bacteriocinogenic cultures and bacteriocins against *L. monocytogenes* in fresh or soft cheeses.

Bacteriocin	Source organism	Mode of utilisation	References
Enterocin 416K1	*Enterococcus casseliflavus*	Bacteriocin entrapped in polymeric film	[46]
Cerein 8A	*Bacillus cereus*	Surface application of the bacteriocin	[47]
Enterocin A and B	*Enterococcus faecium*	Adjunct culture in brine and smearing solution	[48]
Lacticin 3147	*Lactococcus lactis*	Smeared on the cheese surface	[26]
Nisin and Pediocin PA-1	Recombinant *Lactococcus lactis*	Starter culture	[49]
Enterocin A	Recombinant *Lactococcus lactis*	Starter culture	[50]



short Illumina PE reads

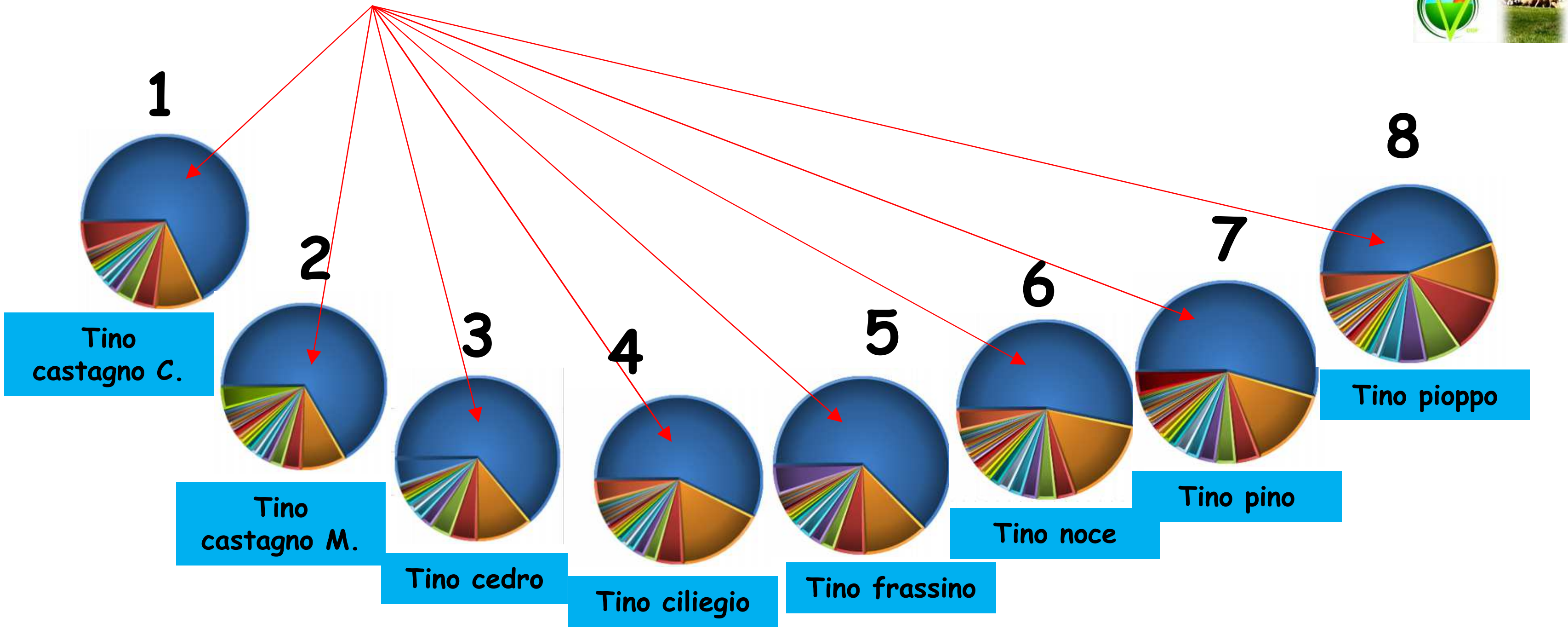
Q-check

Metagenome assembly

Genome/contig binning

Taxonomic classification

Risultati

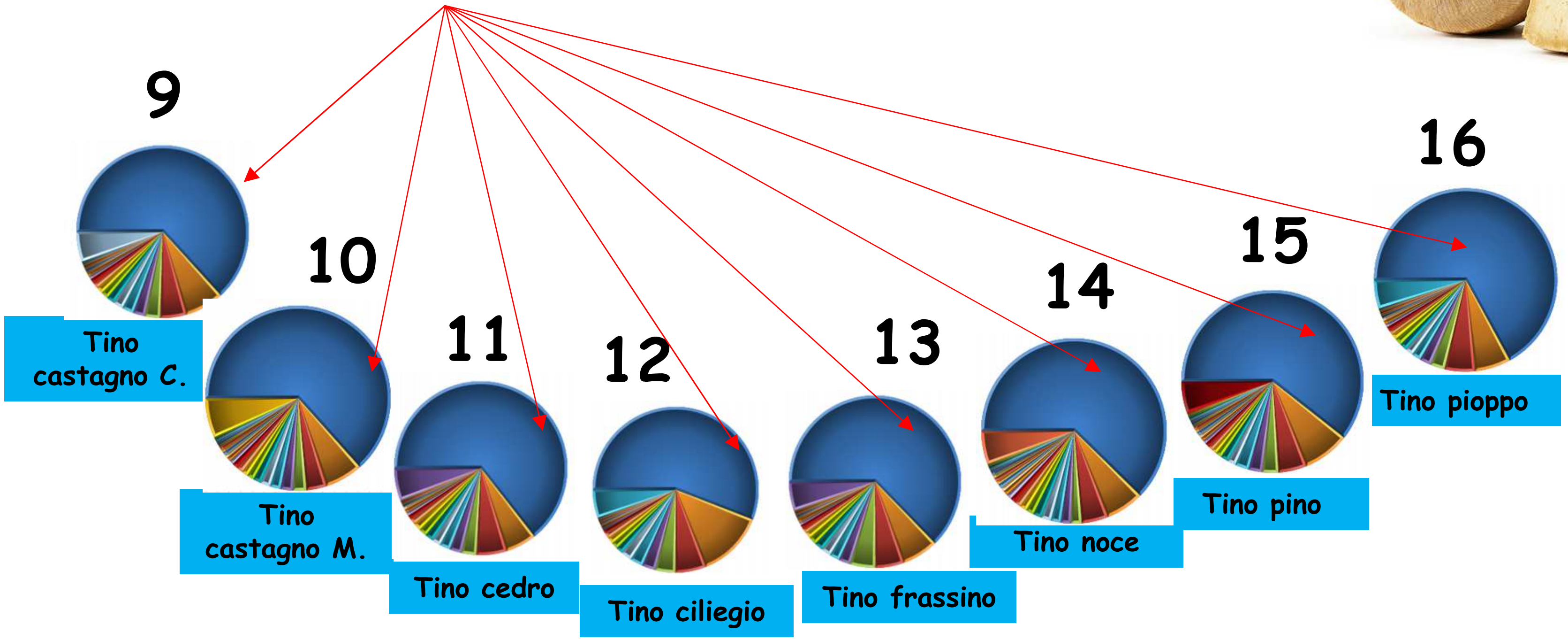


Specie batteriche

- *Streptococcus pluranimalium* (8-17%)
- *Lactobacillus maniotivorans* (3-10%)
- *Lactobacillus brantae* (2-6%)
- *Streptoccoccus bovis, lactococcus lactis, lactobacillus parakefiri* (2-5%)
- *Streptoccoccus equinus, Lactococcus fujiensis, Enterobacter hormaechei, Streptococcus thermophilus* (1-2%)
- Specie batteriche non classificate (40-70%)

Risultati

Specie batteriche



9

Tino castagno C.

10

Tino castagno M.

11

Tino cedro

12

Tino ciliegio

13

Tino frassino

14

Tino noce

15

Tino pino

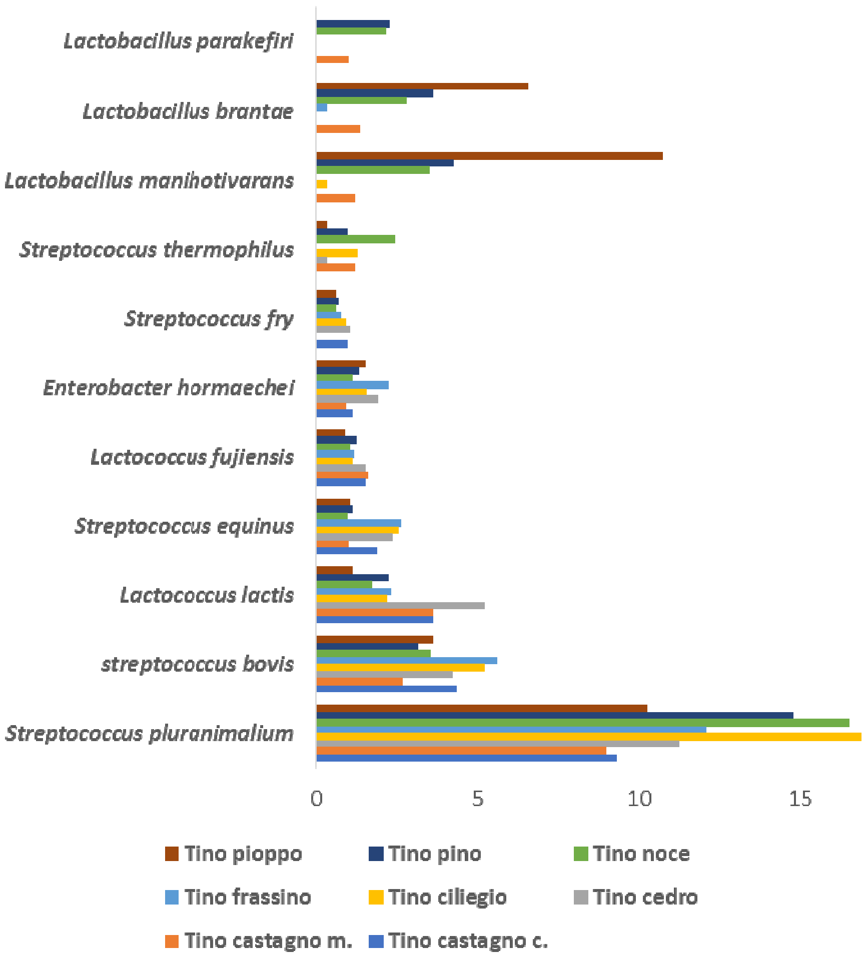
16

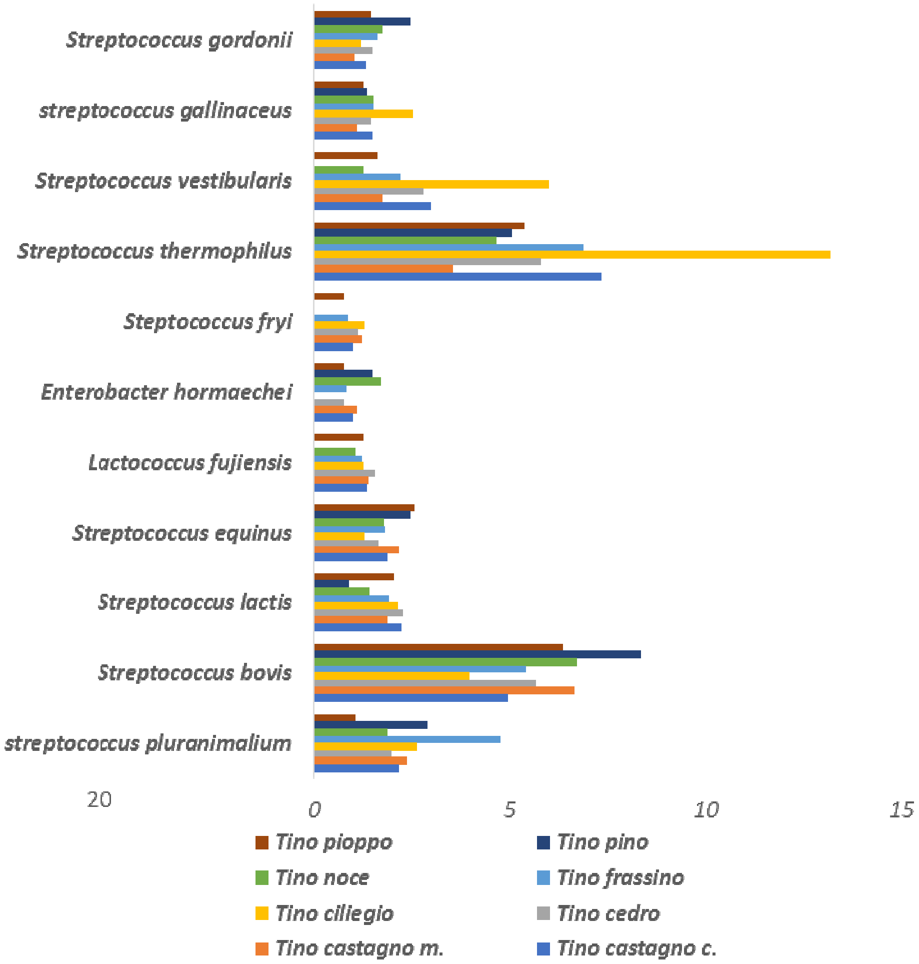
Tino pioppo

- *Streptococcus thermophilus* (4-13%)
- *Streptocccus bovis* (4-8%)
- *Streptococcus vestibularis* (2-6%)
- *Streptococcus pluranimalium (1-4%)*
- *Streptococcus equinus, Streptococcus gallinaceus, S. gordonii, S. fryi (1-2%)*
- *Lactococcus lactis, lactococcus fujiensis* (1-2%)
- Specie batteriche non classificate (40-70%)

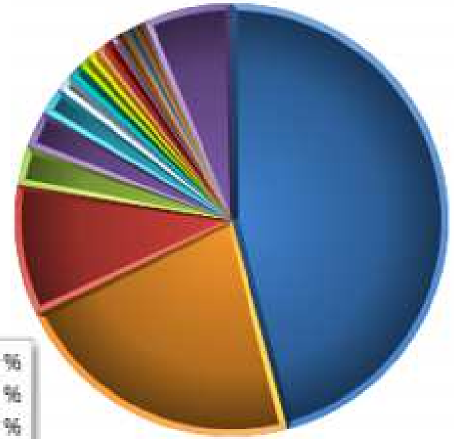
Risultati


Vastedda della valle del Belice
denominazione d'origine protetta

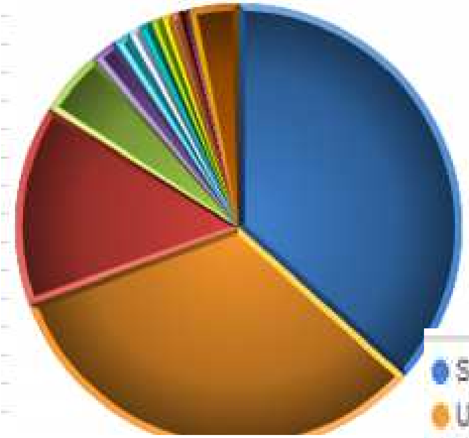

Lactobacillus parakefiri
Lactobacillus brantae
Lactobacillus manihotivarans
Streptococcus thermophilus
Streptococcus fry
Enterobacter hormaechei
Lactococcus fujiensis
Streptococcus equinus
Lactococcus lactis
streptococcus bovis
Streptococcus pluranimalium
0
5
10
15
20
Tino pioppo
Tino pino
Tino noce
Tino frassino
Tino ciliegio
Tino cedro
Tino castagno m.
Tino castagno c.


Streptococcus gordonii
streptococcus gallinaceus
Streptococcus vestibularis
Streptococcus thermophilus
Steptococcus fryi
Enterobacter hormaechei
Lactococcus fujiensis
Streptococcus equinus
Streptococcus lactis
Streptococcus bovis
streptococcus pluranimalium
0
5
10
15
Tino pioppo
Tino pino
Tino noce
Tino frassino
Tino ciliegio
Tino cedro
Tino castagno m.
Tino castagno c.

Risultati



● Unclassified	45.87 %
● Streptococcus pluranimalium	21.92 %
● Streptococcus thermophilus	9.85 %
● Streptococcus vestibularis	2.9 %
● Streptococcus bovis	2.8 %
● Streptococcus gallinaceus	2.05 %
● Streptococcus gordonii	1.23 %
● Lactococcus lactis	1.06 %
● Streptococcus equinus	0.85 %
● Streptococcus fryi	0.8 %
● Lactobacillus rhamnosus	0.75 %
● Lactococcus fujiensis	0.6 %
● Lactobacillus parakefiri	0.52 %
● Acinetobacter johnsonii	0.44 %
● Lactobacillus parabuchneri	0.43 %
● Lactobacillus camelliae	0.42 %
● Streptococcus thoraltensis	0.32 %
● Acinetobacter tjernbergiae	0.31 %
● Streptococcus dentirousetti	0.29 %
● Other Species	6.59 %
Total	100 %

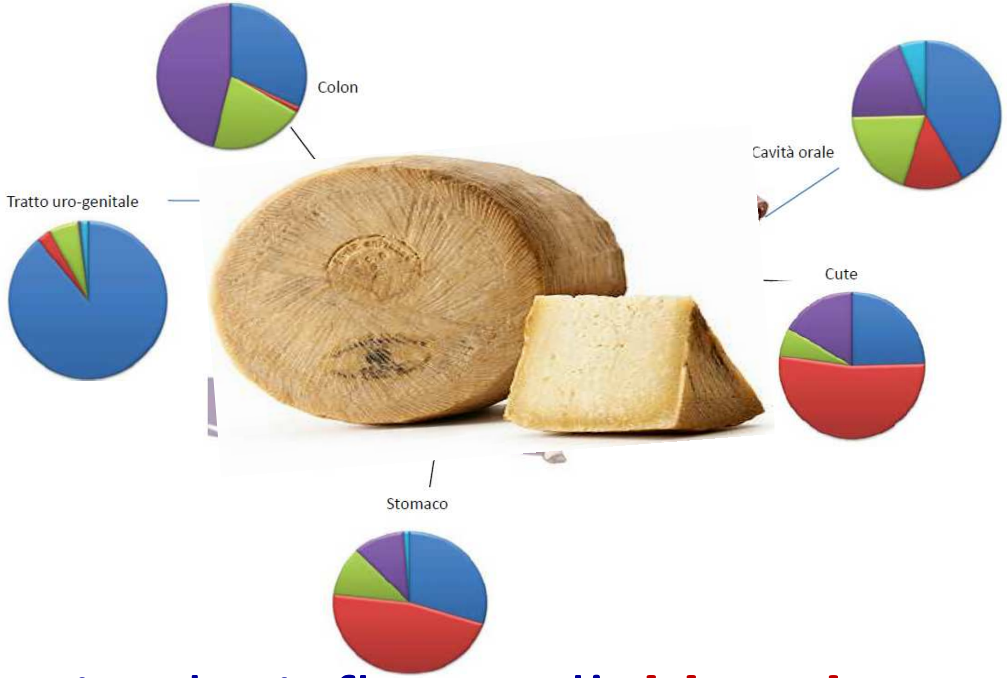


● Streptococcus thermophilus	36.62 %
● Unclassified	32.71 %
● Streptococcus pluranimalium	14.59 %
● Streptococcus gallinaceus	4.84 %
● Streptococcus vestibularis	1.89 %
● Lactobacillus delbrueckii	1.17 %
● Streptococcus fryi	0.89 %
● Streptococcus bovis	0.87 %
● Lactobacillus rhamnosus	0.82 %
● Lactobacillus ultunensis	0.78 %
● Lactobacillus parakefiri	0.53 %
● Lactobacillus camelliae	0.42 %
● Streptococcus milleri	0.31 %
● Other Species	3.56 %
Total	100 %

Complessità del microbiota

- Le comunità batteriche del FORMAGGI appaiono costituite da un “**core microbiota**”

Essenziale per i processi di maturazione



L’alimentazione animale, influenza l’**abbondanza** e la **tipologia** delle OTUs che costituiscono il microbiota del latte e quindi dei formaggi in relazione alla tipologia di lavorazione

Conclusioni

- ✓ Lo studio delle comunità microbiche dei formaggi ha dimostrato che vi è un'abbondante popolazione di batteri la cu presenza è da correlare prevalentemente alle materie prime.
- ✓ L'applicazione delle tecniche di sequenziamento NGS svela la composizione della microflora dei formaggi.
- ✓ La tecnologia NGS potrebbe quindi diventare in futuro uno strumento nella gestione delle filiere produttive nell'industria alimentare per migliorare la qualità e la sicurezza delle produzioni tipiche.

Grazie