



Istituto Zooprofilattico sperimentale della Sicilia



Metagenomica e sanità pubblica veterinaria

Metagenomica di una produzione casearia siciliana

Relatore: Stefano Reale

II° WORKSHOP DEL COMITATO TECNICO PER LA GENOMICA



FORMAGGI

Introduzione

Alimenti: ecosistemi ben definiti, la cui complessità del microbioma è determinata dalle interazioni tra i fattori ambientali estrinseci, ed intrinseci dell'alimento stesso.

Le popolazioni microbiche presenti nell'alimento, possono modificare la loro composizione durante la maturazione, stagionatura o shelf-life, instaurando dei meccanismi di interazione che spesso vengono solo parzialmente evidenziati con i metodi di analisi microbiologica tradizionali.

Durante la stagionatura dei formaggi si succedono varie specie batteriche che portano alla trasformazione biochimica di grassi e proteine, con l'ottenimento delle caratteristiche organolettiche finali.

Il Next Generation Sequencing (NGS) rappresenta uno strumento potente per studiare a fondo le comunità microbiche degli alimenti

E' una tecnica indispensabile per complementare le metodiche coltura-dipendenti della microbiologia, visto che spesso molti microrganismi sono difficilmente isolabili.



La maturazione

Nel corso della maturazione si operano varie trasformazioni dovute a :

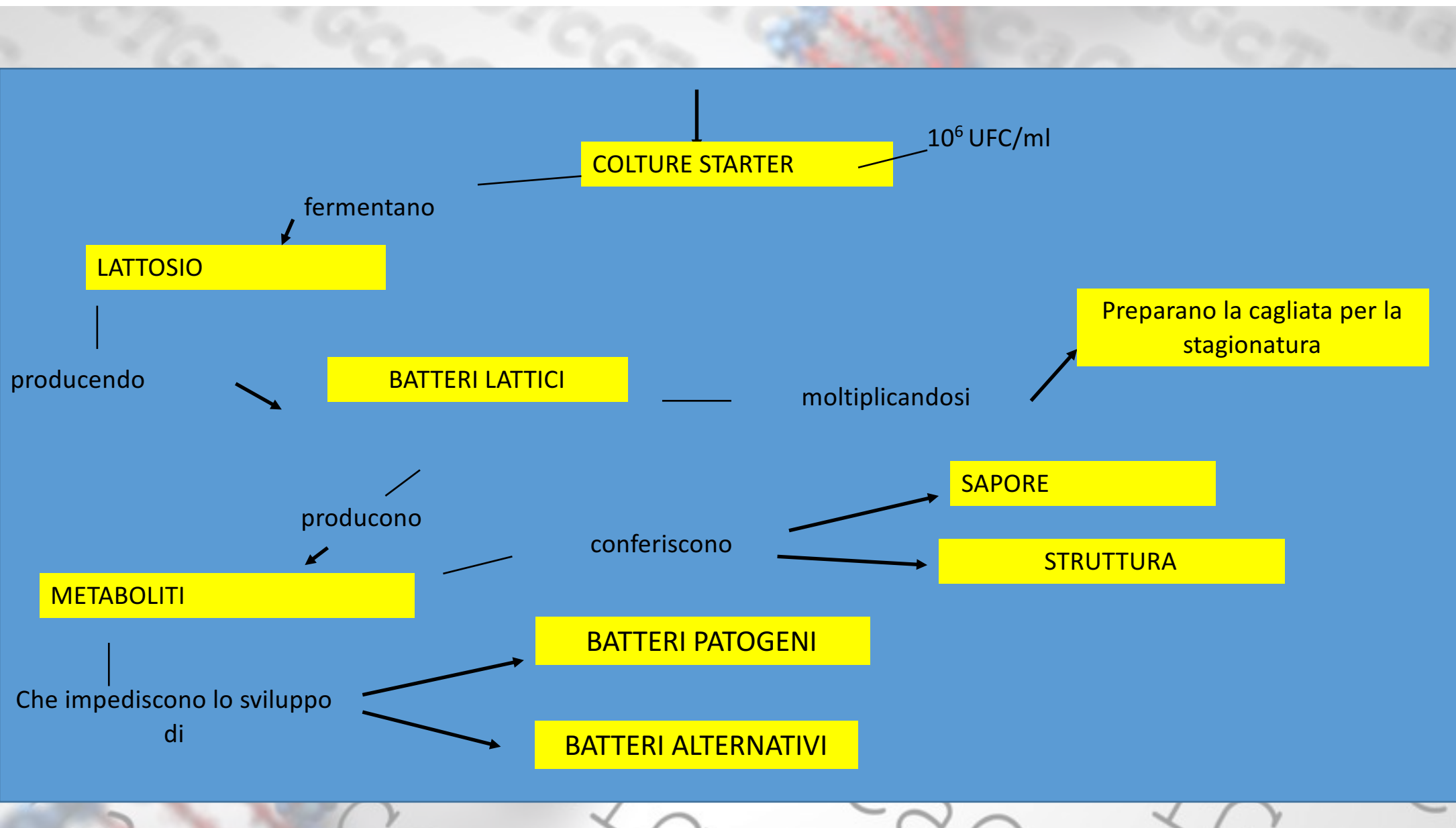
- enzimi del latte (soprattutto nei formaggi a latte crudo)
- attività residua del caglio (chimosina)
- enzimi degli starter batterici (lattici)
- enzimi di microrganismi non starter (starter secondari, muffe, batteri propionici, o non starter già presenti nel latte o a sviluppo successivo).

Tutti questi fenomeni possono essere ricondotti a:

perdita di umidità e essiccamento, con formazione della crosta;
glucidolisi;
proteolisi;
idrolisi del grasso.

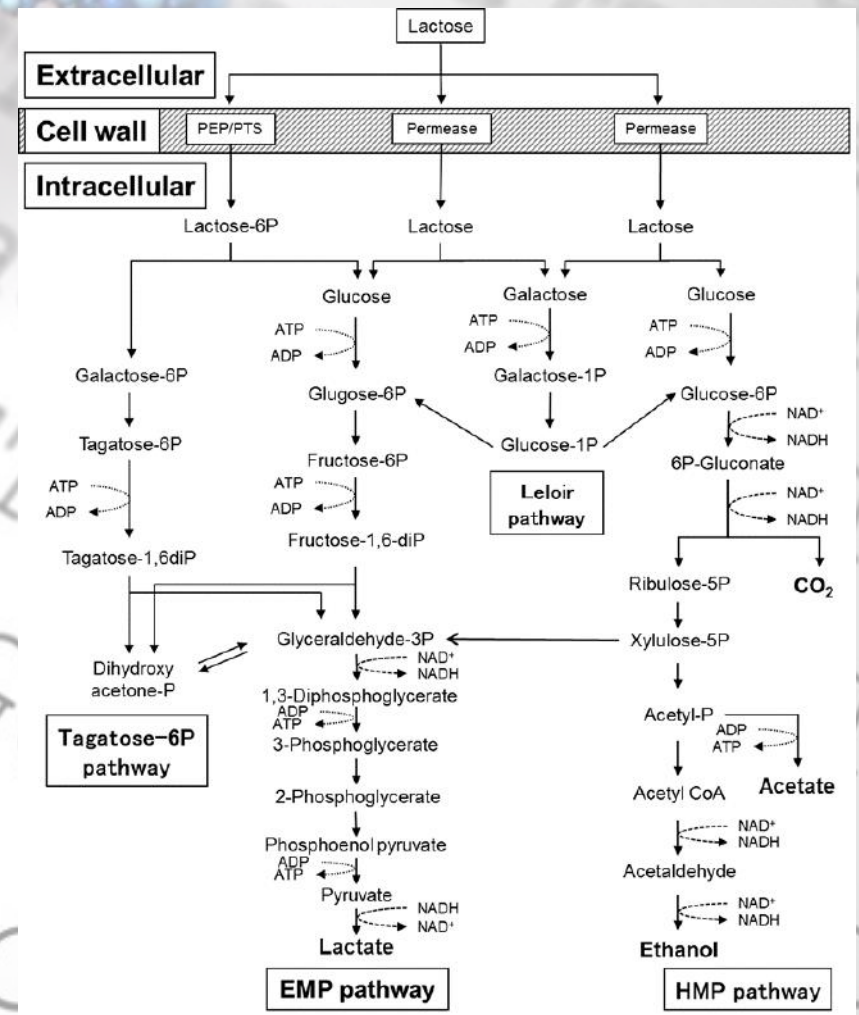
Ruolo DEL MICROBIOMA:

- 1 .Metabolismo Primario funzionale al prodotto (es. acidificazione latte per formaggio)
- 2.Inibizione alterativi e patogeni (competizione nutrizionale; produzione sostanze a diverso spettro)
- 3.Metabolismo Secondario. (Produzione sostanze aromatiche –stagionatura: maturazione)
- 4.Autolisi e rilascio enzimi intracellulari
5. Fase di maturazione



glucidolisi

- idrolisi del lattosio
- fermentazione ad acido lattico
seguito da neutralizzazione dello stesso
- scomparsa dell'acido lattico
- aumento del pH;



idrolisi del grasso

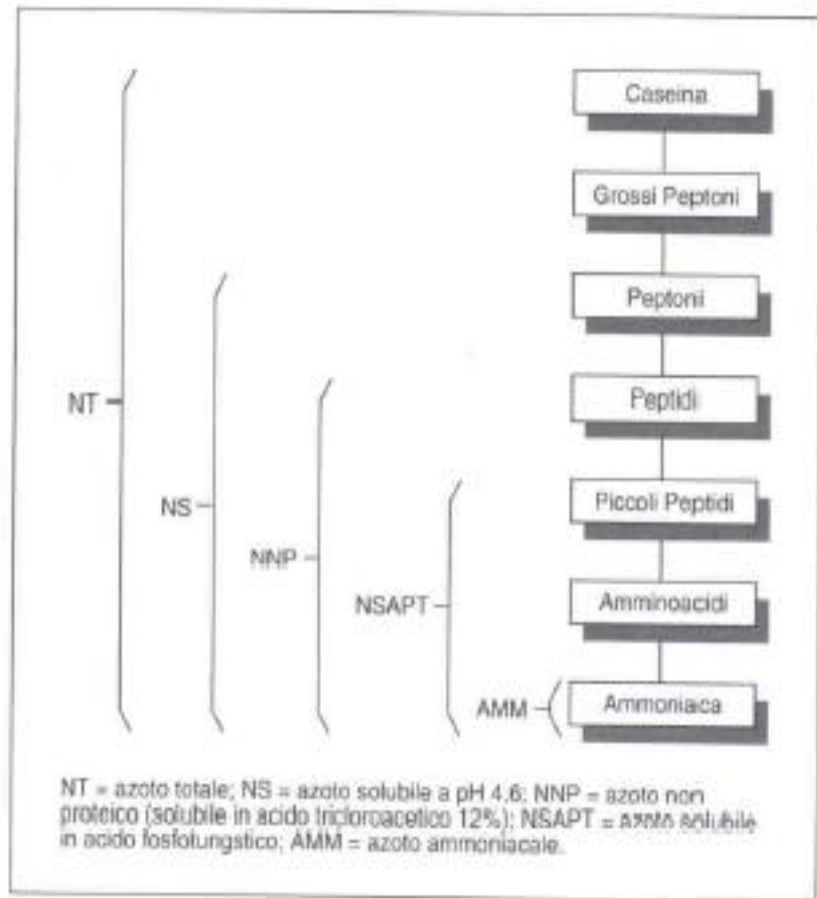
Dalla **frazione lipidica** si possono formare vari prodotti intermedi di idrolisi dei trigliceridi:

- digliceridi,
- monogliceridi,
- glicerolo
- acidi grassi liberi;

questi ultimi possono essere ulteriormente degradati a

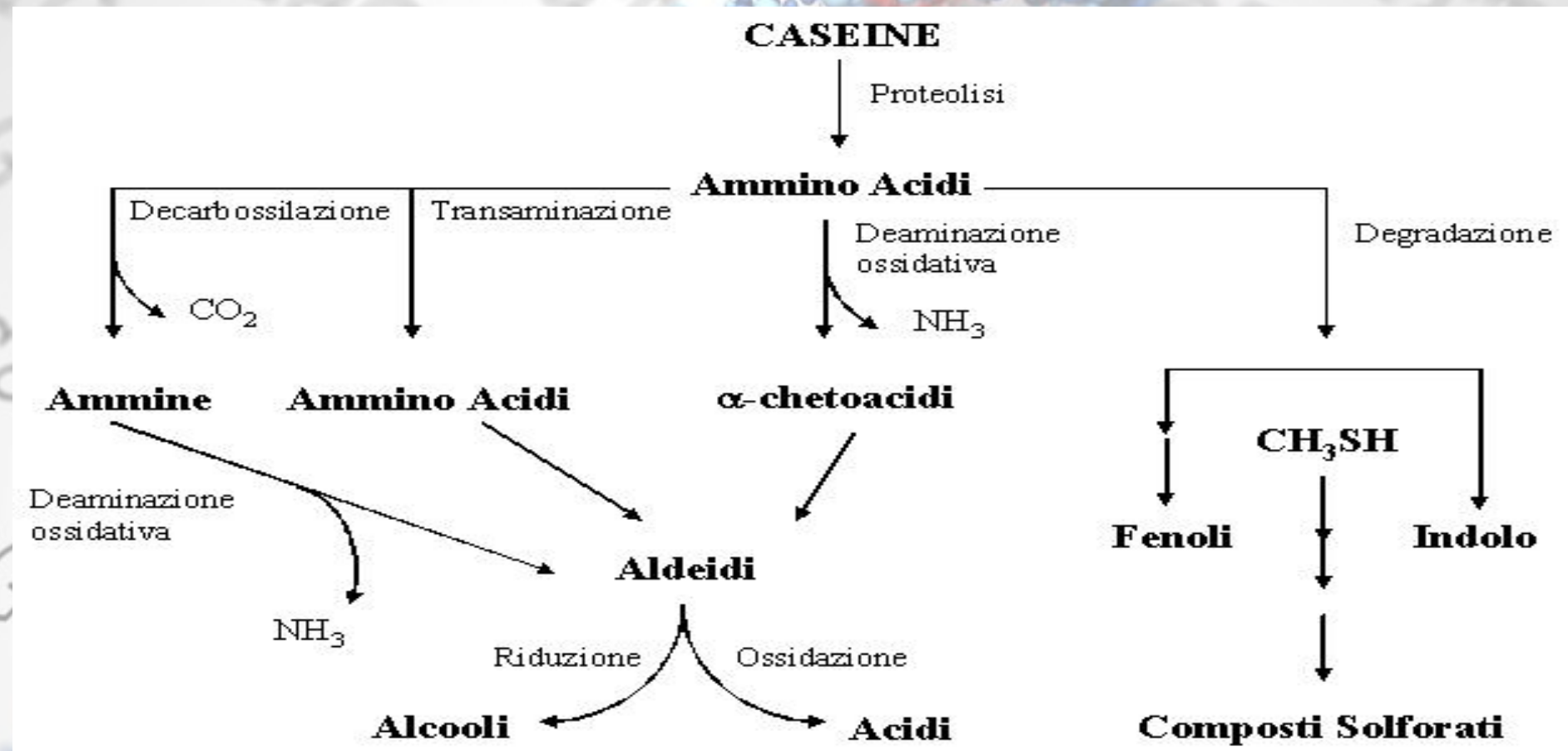
- aldeidi,
- alcoli,
- γ -lattoni,
- δ -lattoni
- metilchetoni.

proteolisi



<i>Lactococcus</i>	Acido glutammico, valina, metionina, leucina, isoleucina, istidina e spesso anche fenilalanina, tiroxina, lisina e alanina (5).
<i>Leuconostoc</i>	Hanno esigenze molto variabili a seconda della specie: in genere acido glutammico e metionina sono stimolanti dello sviluppo.
<i>Streptococcus</i>	Acido glutammico, istidina, cisteina, metionina, valina, leucina, triptofano e tirosina.
<i>Lactobacillus</i>	Manifestano esigenze molto variabili fra le varie specie: per <i>L. helveticus</i> sono richiesti acido glutammico, alanina prolina e serina mentre per <i>L. delbrueckij</i> subsp. <i>Lactis</i> e <i>L. casei</i> subsp. <i>casei</i> è richiesta treonina.

IDROLISI DELLE PROTEINE



Lactococcus lactis subsp. *lactis*
Lactococcus lactis subsp. *diacetylactis*
Lactococcus lactis subsp. *cremoris*
Leuconostoc mesenteroides subsp. *cremoris*
Leuconostoc lactis
Lactobacillus acidophilus
Lactobacillus casei subsp. *casei*
Lactobacillus casei subsp. *rhamnosus*
Lactobacillus casei subsp. *pseudoplatarum*

BATTERI
LATTICI
MESOFILI

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

BATTERI
LATTICI
TERMOFILI

Microrganismi non lattici di interesse lattiero caseario

- Clostridi
- Batteri propionici
- Bifidobatteri
- Pseudomonas
- Cromobatteri
- Micrococchi
- Bacillus
- Colibatteri
- Lieviti
- Muffe



CONTAMINANTI

Streptococchi fecali, *S. specie* minori

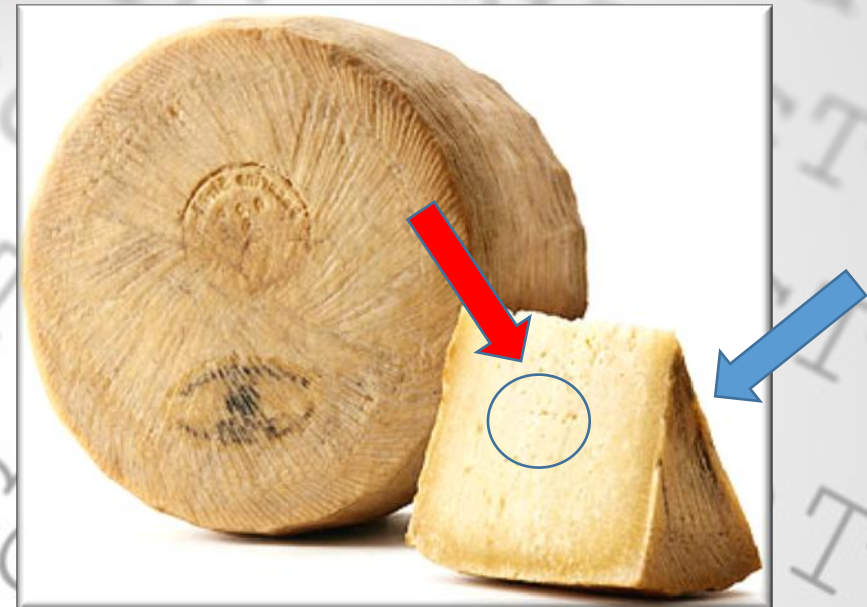


FERMENTAZIONE

- **LATTICA** acido lattico aroma di formaggio fresco
- **PROPIONICA** acido lattico dal lattosio tipico aroma dei formaggi cotti;
 - Propionibacterium spp. acido propionico ed formaggi occhiati.
- **anaerobi** acetico dai lattati; CO₂
- **BUTIRRICA** acido acetico e butirrico dai lattati; aroma putrido,
 - **Clostridium butiricum**, CO₂ e idrogeno gusto piccante; C. tyrobutiricum gonfiore tardivo dei formaggi duri
- **ALCOLICA** Produzione di glucosio e galattosio; utili nella stagionatura
 - Lieviti che idrolizzano etanolo e composti aromatici gonfiore precoce dei formaggi;
- **da enterobatteri**
 - lattosio: acido lattico, aromi particolari
- - acido acetico, idrogeno e CO₂

Obiettivi dello studio

- Studio preliminare del microbiota di un formaggio tipico siciliano (Pecorino siciliano)
- Analisi differenziale della popolazione batterica tra la parte **ESTERNA** ed **INTERNA** di due forme di formaggio F1 e F2 delle quali:
 - F1. buona produzione (commestibile)
 - F2. cattiva produzione (non commestibile, con bolle)



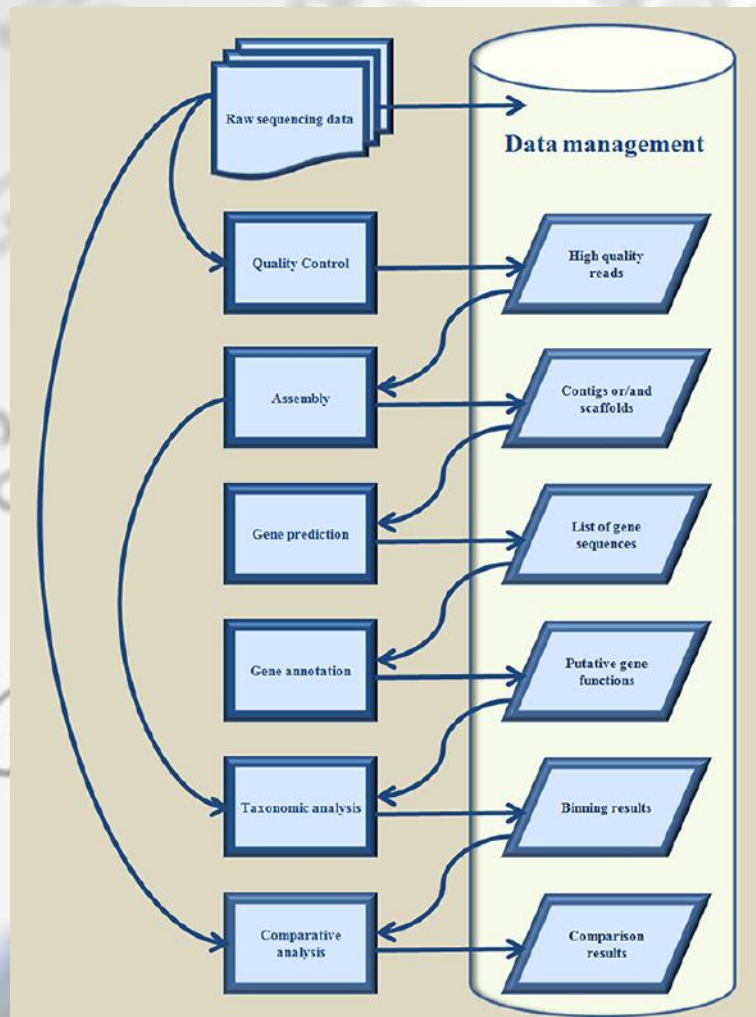
Workflow

- **Campionamento**
- **Estrazione del DNA:**
Kit MERICON (Qiagen) in doppio
- **Librerie genomiche:**
Nextera XT
Agencourt AMPure XP
- **Sequenziatore NGS:**
MiSeq Illumina
- **Analisi bioinformatica:**
BaseSpace



BaseSpace
SEQUENCE HUB

Integrative workflows for metagenomic analysis



BIOINFORMATIC ARSENAL

- (i) CloVR-metagenomics ([Angiuoli et al., 2011](#)),
- (ii) Galaxy platform (metagenomics pipeline) ([Giardine et al., 2005](#)),
- (iii) IMG/M ([Markowitz et al., 2008, 2014](#)),
- (iv) MetAMOS ([Treangen et al., 2013](#)),
- (v) MG-RAST ([Aziz et al., 2008](#); [Meyer et al., 2008](#)),
- (vi) RAMMCPAP ([Li, 2009](#)),
- (vii) SmashCommunity ([Arumugam et al., 2010](#)).

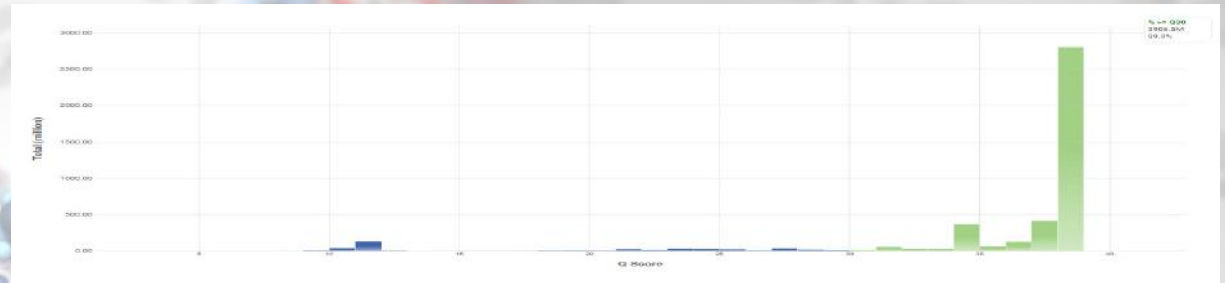
PIPELINES

Table 1 | Display of features of current bioinformatic pipelines for metagenomic data analysis.

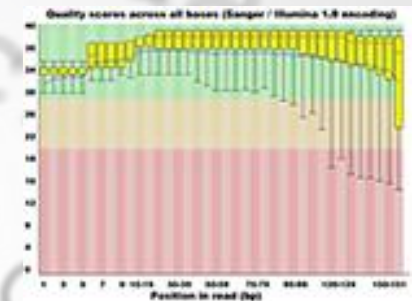
Pipeline Tasks	Quality control	Assembly	Gene detection	Functional annotation	Taxonomic analysis	Comparative analysis	Data management
CloVR-metagenomics	✗	✗	✓	✓	✓	✓	✓
Galaxy platform*	✓	✗	✗	✗	✓	✓	✗
IMG/M	✗	✗	✓	✓	✓	✓	✓
MetAMOS	✓	✓	✓	✓	✓	✓	✓
MG-RAST	✗	✗	✓	✓	✓	✓	✓
RAMMCP	✗	✗	✓	✓	✓	✓	✗
SmashCommunity	✓	✓	✓	✓	✓	✓	✓

Risultati

Aggregate results



Sample Number	Number Reads PF	% Reads PF Classified to Genus	Number of Species Identified
1	1,471,779	92.46%	787
2	1,857,512	91.55%	770
3	1,777,784	91.93%	789
4	1,430,781	91.46%	802
5	1,900,919	89.13%	652
6	1,481,703	90.71%	683
7	1,542,804	90.88%	702
8	1,173,598	90.09%	694



Risultati

Crosta F1

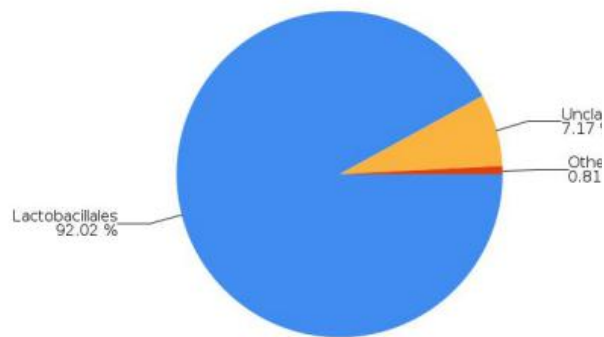
Top Order Classification Results

Classification	Number of Reads	% Total Reads
Lactobacillales	1,079,994	92.02 %
Unclassified at Order level	84,136	7.17 %
Clostridiales	2,086	0.18 %
Bacillales	2,081	0.18 %
Enterobacteriales	2,063	0.18 %
Aeromonadales	765	0.07 %
Actinomycetales	560	0.05 %
Pseudomonadales	325	0.03 %

Total Order-level Taxonomic Categories Identified: 64. This table shows the top 8 of 64 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Order Classification Results



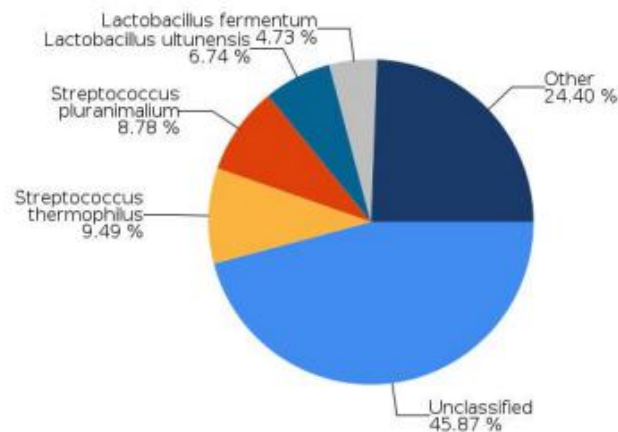
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	538,293	45.87 %
Streptococcus thermophilus	111,325	9.49 %
Streptococcus pluranimalium	103,010	8.78 %
Lactobacillus ultunensis	79,104	6.74 %
Lactobacillus fermentum	55,538	4.73 %
Lactobacillus delbrueckii	39,729	3.39 %
Streptococcus gallinaceus	39,005	3.32 %
Streptococcus vestibularis	26,094	2.22 %

Total Species-level Taxonomic Categories Identified: 595. This table shows the top 8 of 595 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



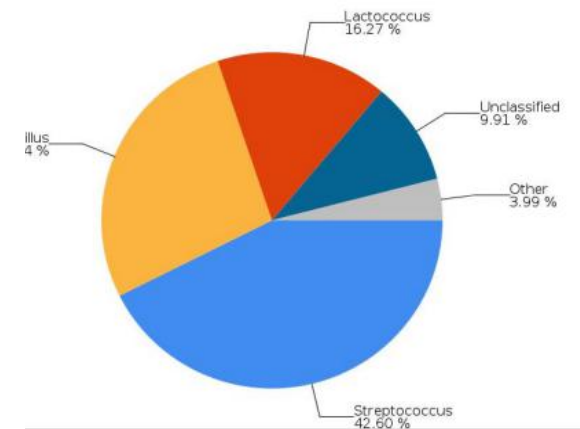
Top Genus Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Genus level	499,946	42.60 %
Streptococcus	319,636	27.24 %
Lactococcus	190,901	16.27 %
Other	116,271	9.91 %
Lactobacillus	22,035	1.88 %
Other	6,765	0.58 %
Other	3,326	0.28 %
Other	2,258	0.19 %

Total Genus-level Taxonomic Categories Identified: 255. This table shows the top 8 of 255 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Genus Classification Results



Risultati

Crosta F2

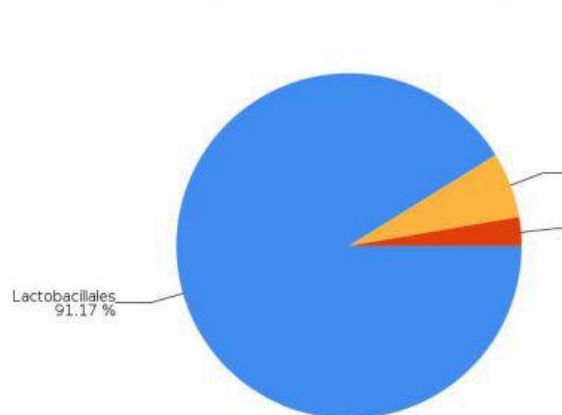
Top Order Classification Results

Classification	Number of Reads	% Total Reads
Lactobacillales	1,341,870	91.17 %
Unclassified at Order level	90,466	6.15 %
Enterobacterales	12,154	0.83 %
Pseudomonadales	7,359	0.50 %
Bacillales	6,905	0.47 %
Actinomycetales	5,250	0.36 %
Aeromonadales	4,391	0.30 %
Clostridiales	1,190	0.08 %

Total Order-level Taxonomic Categories Identified: 71. This table shows the top 8 of 71 categories.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Order Classification Results



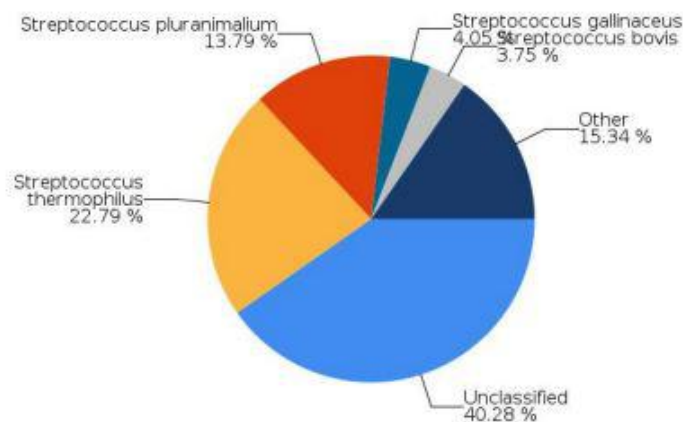
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	592,772	40.28 %
Streptococcus thermophilus	335,413	22.79 %
Streptococcus pluranimalium	203,007	13.79 %
Streptococcus gallinaceus	59,592	4.05 %
Streptococcus bovis	55,196	3.75 %
Streptococcus vestibularis	36,926	2.51 %
Streptococcus gordonii	22,654	1.54 %
Streptococcus fryi	12,668	0.86 %

Total Species-level Taxonomic Categories Identified: 688. This table shows the top 8 of 688 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



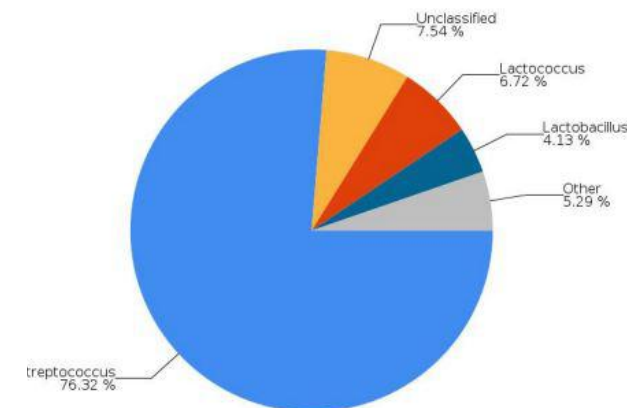
Top Genus Classification Results

Classification	Number of Reads	% Total Reads
Streptococcus	1,123,289	76.32 %
Unclassified at Genus level	111,011	7.54 %
Lactococcus	98,878	6.72 %
Lactobacillus	60,765	4.13 %
Other	17,905	1.22 %
Streptococcus	10,628	0.72 %
Streptococcus	8,084	0.55 %
Streptococcus	6,900	0.47 %

Total Genus-level Taxonomic Categories Identified: 303. This table shows the top 8 of 303 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Genus Classification Results



Risultati

Confronto tra i campioni Crosta F1 - F2



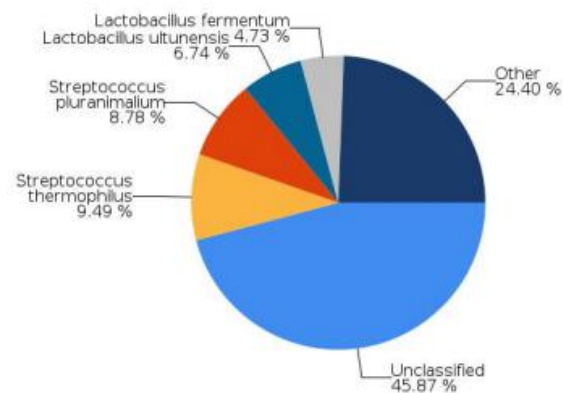
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	538,293	45.87 %
Streptococcus thermophilus	111,325	9.49 %
Streptococcus pluranimalium	103,010	8.78 %
Lactobacillus ultunensis	79,104	6.74 %
Lactobacillus fermentum	55,538	4.73 %
Lactobacillus delbrueckii	39,729	3.39 %
Streptococcus gallinaceus	39,005	3.32 %
Streptococcus vestibularis	26,094	2.22 %

Total Species-level Taxonomic Categories Identified: 595. This table shows the top 8 of 595 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



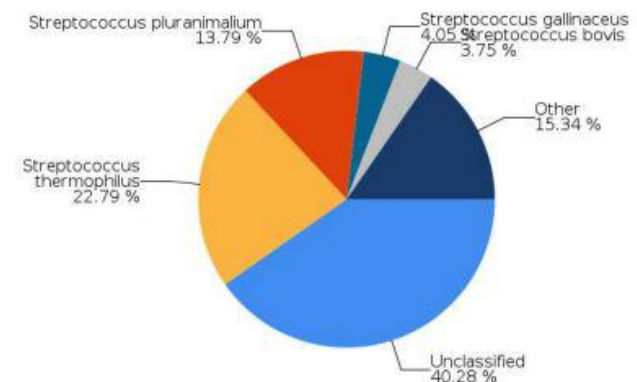
Top Species Classification Results

Classification	Number of Reads	% Total Reads
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Streptococcus thermophilus	335,413	22.79 %
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Streptococcus vestibularis	36,926	2.51 %
Streptococcus gordonii	22,654	1.54 %
Streptococcus fryi	12,668	0.86 %

Total Species-level Taxonomic Categories Identified: 688. This table shows the top 8 of 688 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



Risultati

Cuore F1

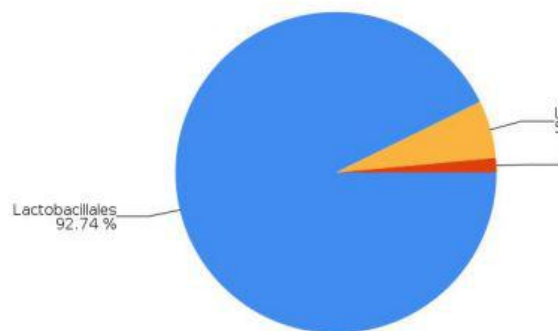
Top Order Classification Results

Classification	Number of Reads	% Total Reads
Lactobacillales	1,430,737	92.74 %
Unclassified at Order level	89,591	5.81 %
Enterobacteriales	8,308	0.54 %
Clostridiales	3,030	0.20 %
Bacillales	2,901	0.19 %
Aeromonadales	2,548	0.17 %
Pseudomonadales	1,840	0.12 %
Flavobacteriales	861	0.06 %

Total Order-level Taxonomic Categories Identified: 68. This table shows the top 8 of 68 cl

Note: The "Other" category in this pie chart is the sum of all classifications with less than

Top Order Classification Results



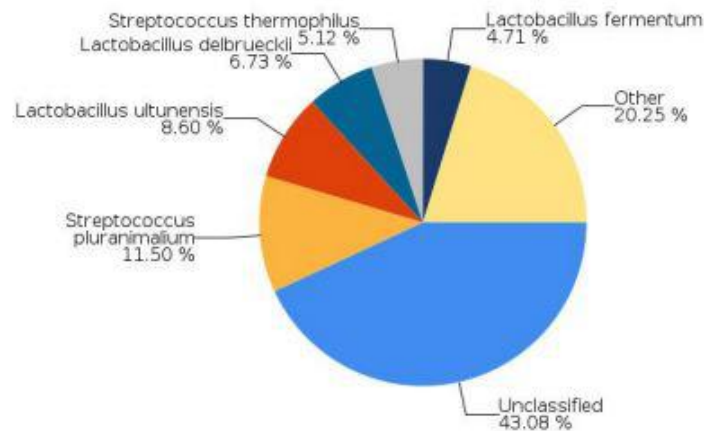
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	664,654	43.08 %
Streptococcus pluranimalium	177,495	11.50 %
Lactobacillus ultunensis	132,735	8.60 %
Lactobacillus delbrueckii	103,770	6.73 %
Streptococcus thermophilus	79,032	5.12 %
Lactobacillus fermentum	72,708	4.71 %
Streptococcus gallinaceus	34,159	2.21 %
Lactobacillus helveticus	25,054	1.62 %

Total Species-level Taxonomic Categories Identified: 703. This table shows the top 8 of 703 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



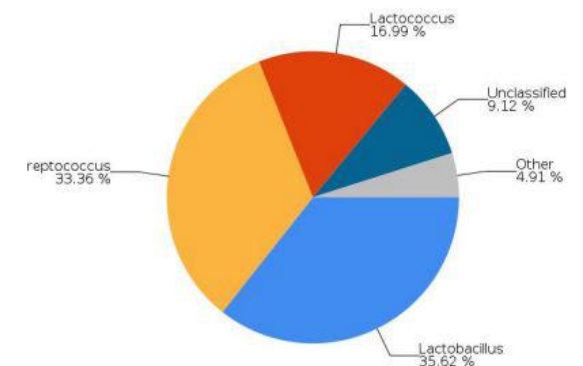
Top Genus Classification Results

Classification	Number of Reads	% Total Reads
Lactococcus	549,611	35.62 %
Streptococcus	514,718	33.36 %
Lactobacillus	262,081	16.99 %
Unclassified at Genus level	140,715	9.12 %
Other	30,569	1.98 %
Streptococcus	10,729	0.70 %
Other	4,531	0.29 %
Other	4,054	0.26 %

Total Genus-level Taxonomic Categories Identified: 300. This table shows the top 8 of 300 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Genus Classification Results



Risultati

Cuore F2

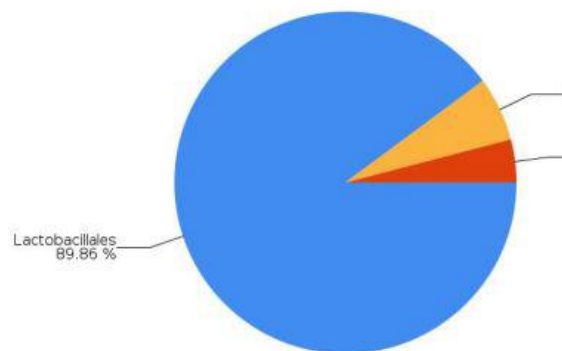
Top Order Classification Results

Classification	Number of Reads	% Total Reads
Lactobacillales	1,669,223	89.86 %
Unclassified at Order level	112,820	6.07 %
Enterobacteriales	37,731	2.03 %
Aeromonadales	9,003	0.48 %
Pseudomonadales	8,027	0.43 %
Actinomycetales	5,652	0.30 %
Bacillales	4,705	0.25 %
Bifidobacteriales	4,566	0.25 %

Total Order-level Taxonomic Categories Identified: 78. This table shows the top 8 of 78 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Order Classification Results



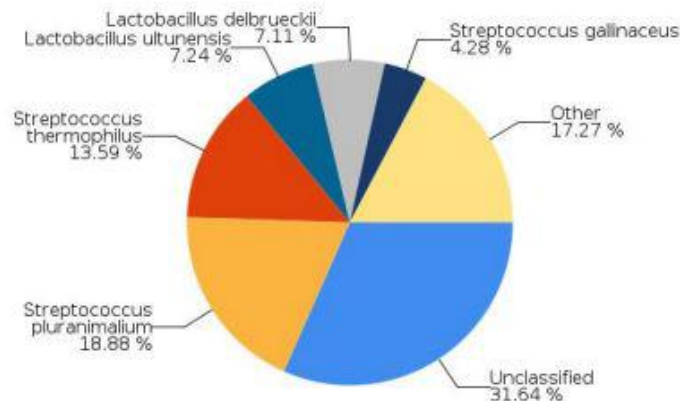
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	587,645	31.64 %
Streptococcus pluranimalium	350,659	18.88 %
Streptococcus thermophilus	252,401	13.59 %
Lactobacillus ultunensis	134,391	7.24 %
Lactobacillus delbrueckii	132,099	7.11 %
Streptococcus gallinaceus	79,523	4.28 %
Lactobacillus helveticus	38,850	2.09 %
Streptococcus bovis	31,520	1.70 %

Total Species-level Taxonomic Categories Identified: 771. This table shows the top 8 of 771 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



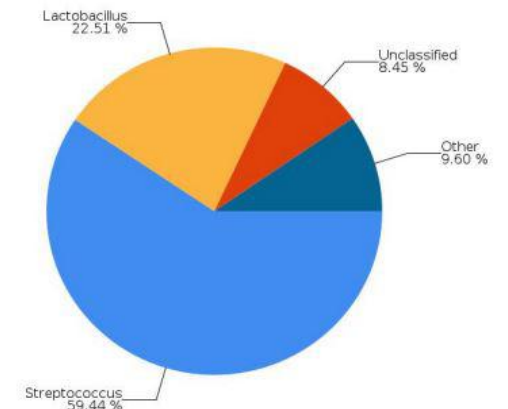
Top Genus Classification Results

Classification	Number of Reads	% Total Reads
Streptococcus	1,104,020	59.44 %
Lactobacillus	418,206	22.51 %
Unclassified at Genus level	156,967	8.45 %
Lactococcus	49,922	2.69 %
Streptococcus	30,044	1.62 %
Streptococcus	28,911	1.56 %
Lactobacillus	10,474	0.56 %
Streptococcus	9,951	0.54 %

Total Genus-level Taxonomic Categories Identified: 358. This table shows the top 8 of 358 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Genus Classification Results



Risultati

Confronto tra i campioni Cuore F1 - F2



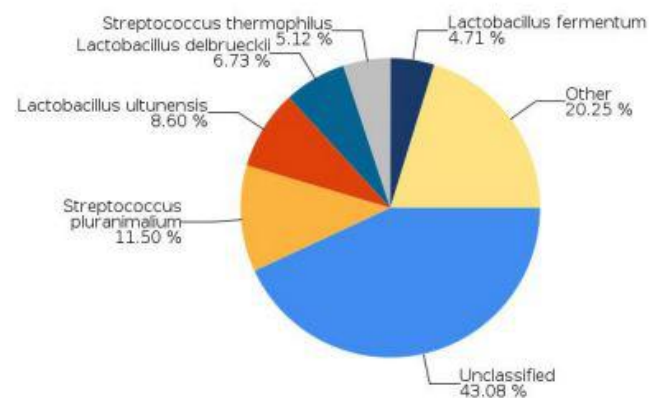
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	664,654	43.08 %
<i>Streptococcus pluranimalium</i>	177,495	11.50 %
<i>Lactobacillus ultunensis</i>	132,735	8.60 %
<i>Lactobacillus delbrueckii</i>	103,770	6.73 %
<i>Streptococcus thermophilus</i>	79,032	5.12 %
<i>Lactobacillus fermentum</i>	72,708	4.71 %
<i>Streptococcus gallinaceus</i>	34,159	2.21 %
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Total Species-level Taxonomic Categories Identified: 703. This table shows the top 8 of 703 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



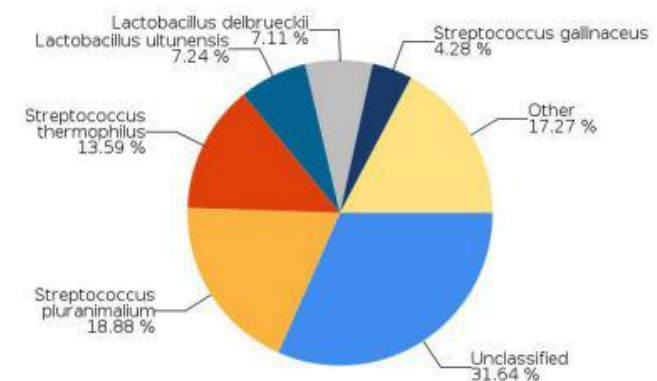
Top Species Classification Results

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<i>Streptococcus gallinaceus</i>	79,523	4.28 %
<i>Lactobacillus helveticus</i>	38,850	2.09 %
<i>Streptococcus bovis</i>	31,520	1.70 %

Total Species-level Taxonomic Categories Identified: 771. This table shows the top 8 of 771 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



Risultati

Crosta - Cuore F1

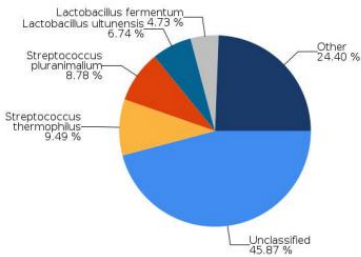
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	538,293	45.87 %
Streptococcus thermophilus	111,325	9.49 %
Streptococcus pluranimalium	103,010	8.78 %
Lactobacillus ultunensis	79,104	6.74 %
Lactobacillus fermentum	55,538	4.73 %
Lactobacillus delbrueckii	39,729	3.39 %
Streptococcus gallinaeus	39,005	3.32 %
Streptococcus vestibularis	26,094	2.22 %

Total Species-level Taxonomic Categories Identified: 595. This table shows the top 8 of 595 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



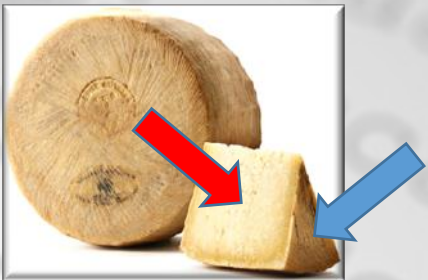
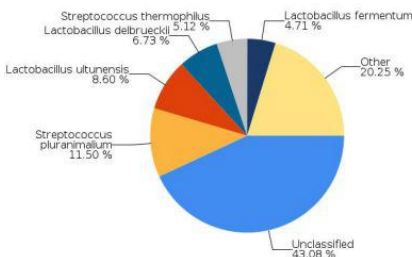
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	664,654	43.08 %
Streptococcus pluranimalium	177,495	11.50 %
Lactobacillus ultunensis	132,735	8.60 %
Lactobacillus delbrueckii	103,770	6.73 %
Streptococcus thermophilus	79,032	5.12 %
Lactobacillus fermentum	72,708	4.71 %
Streptococcus gallinaeus	34,159	2.21 %
Lactobacillus helveticus	25,054	1.62 %

Total Species-level Taxonomic Categories Identified: 703. This table shows the top 8 of 703 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



Crosta - Cuore F2

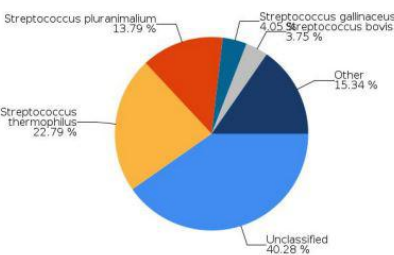
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	592,772	40.28 %
Streptococcus thermophilus	335,413	22.79 %
Streptococcus pluranimalium	203,007	13.79 %
Streptococcus gallinaeus	59,592	4.05 %
Streptococcus bovis	55,196	3.75 %
Streptococcus vestibularis	36,926	2.51 %
Streptococcus gordonii	22,654	1.54 %
Streptococcus fryi	12,668	0.86 %

Total Species-level Taxonomic Categories Identified: 688. This table shows the top 8 of 688 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



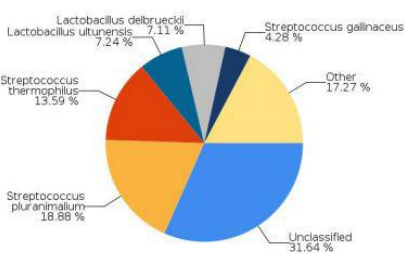
Top Species Classification Results

Classification	Number of Reads	% Total Reads
Unclassified at Species level	587,645	31.64 %
Streptococcus pluranimalium	350,659	18.88 %
Streptococcus thermophilus	252,401	13.59 %
Lactobacillus ultunensis	134,391	7.24 %
Lactobacillus delbrueckii	132,099	7.11 %
Streptococcus gallinaeus	79,523	4.28 %
Lactobacillus helveticus	38,850	2.09 %
Streptococcus bovis	31,520	1.70 %

Total Species-level Taxonomic Categories Identified: 771. This table shows the top 8 of 771 classifications.

Note: The "Other" category in this pie chart is the sum of all classifications with less than 3.50 % abundance.

Top Species Classification Results



Conclusioni

- La parte esterna di F2 è più ricca di batteri contaminanti rispetto a F1, probabilmente causa della cattiva maturazione.



- La parte core di F2 è anch'essa ricca di contaminanti che hanno ostacolato l'opera dei caseificanti che risultano comunque presenti ma in minor numero di specie rispetto alla produzione F1.

Conclusioni

- STANDARDIZZAZIONE
- OTTIMIZZAZIONE DEGLI STARTER
- EVENTUALE BREVETTO PER STARTER DI PRODUZIONI TIPICHE
- ANALISI HACCP LUNGO LE FILIERE
- MIGLIORAMENTO DI FILIERA
- CONTROLLO AMBIENTALE
- MIGLIORAMENTO DELLE PRODUZIONI TIPICHE PER ARRIVARE AL 100% DI SUCCESSO