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Sviluppo di batteri lattici da biofilm di “tine” di legno, incubati in latte alle condizioni di produzione del Ragusano.

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CoRFiLaC, Regione Siciliana, Ragusa, Italia1, DiGeSA, Università di Catania Italia2, DISPA, Università di Catania Italia, Italia3.

INTRODUCTION

Ragusano cheese is a Protected Denomination of Origin (PDO) cheese produced in the Hyblean region of Sicily from raw milk using traditional wood tools, without commercial starters.

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PDO Ragusano cheese production regulation provides for using traditional wooden vat (*"tina"*) in the cheese making process.

"tina" **owns native biofilm that acts as natural inoculation system and represents a valuable source of biodiversity.**

INTRODUCTION

In the manufacture of this brine-salted pasta filata cheese, raw milk is directly placed in a traditional wooden vat (tina) for cheese making and lactic acid is produced by natural milk flora and desirable flora from the biofilm of the surface of the Tina (Lortal et al., 2009).

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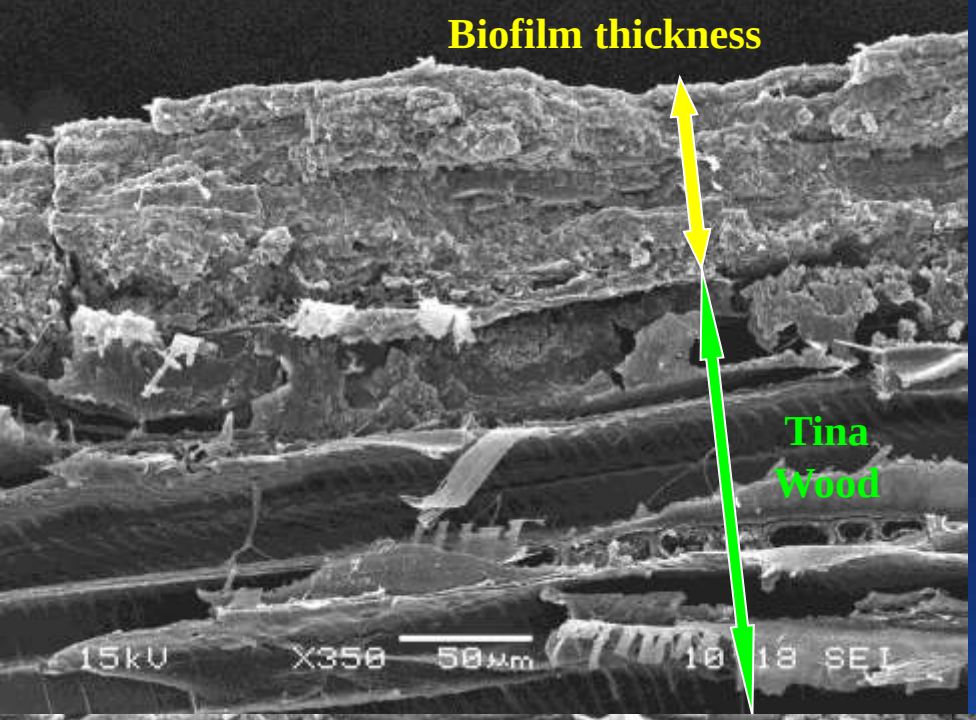
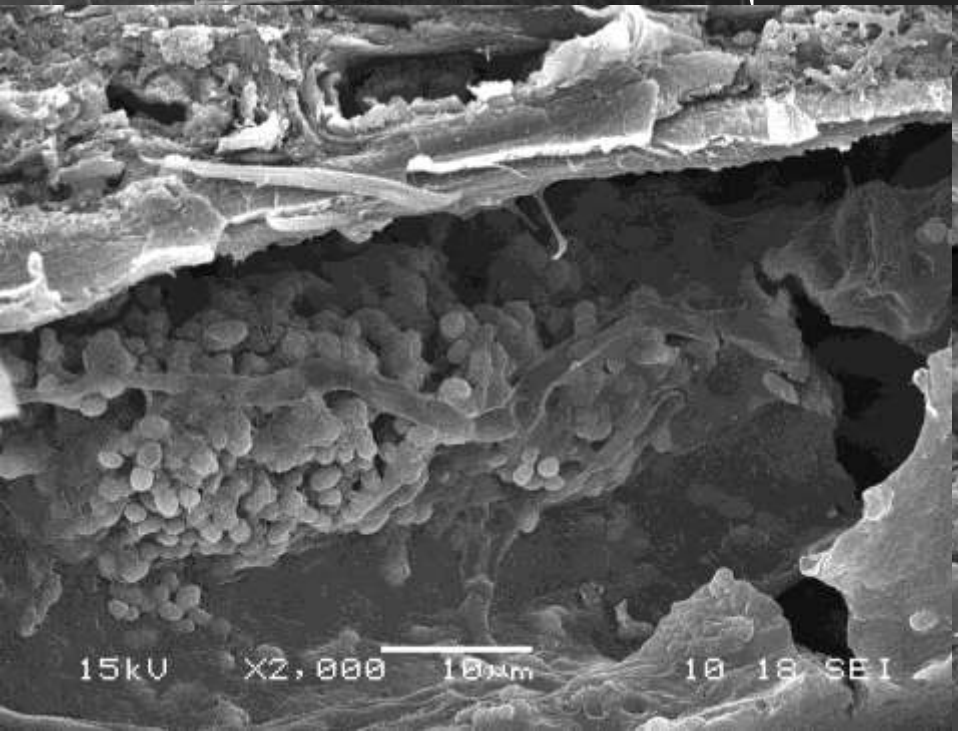

Biofilm thickness
Tina Wood
15kV X350 50µm 10 18 SEI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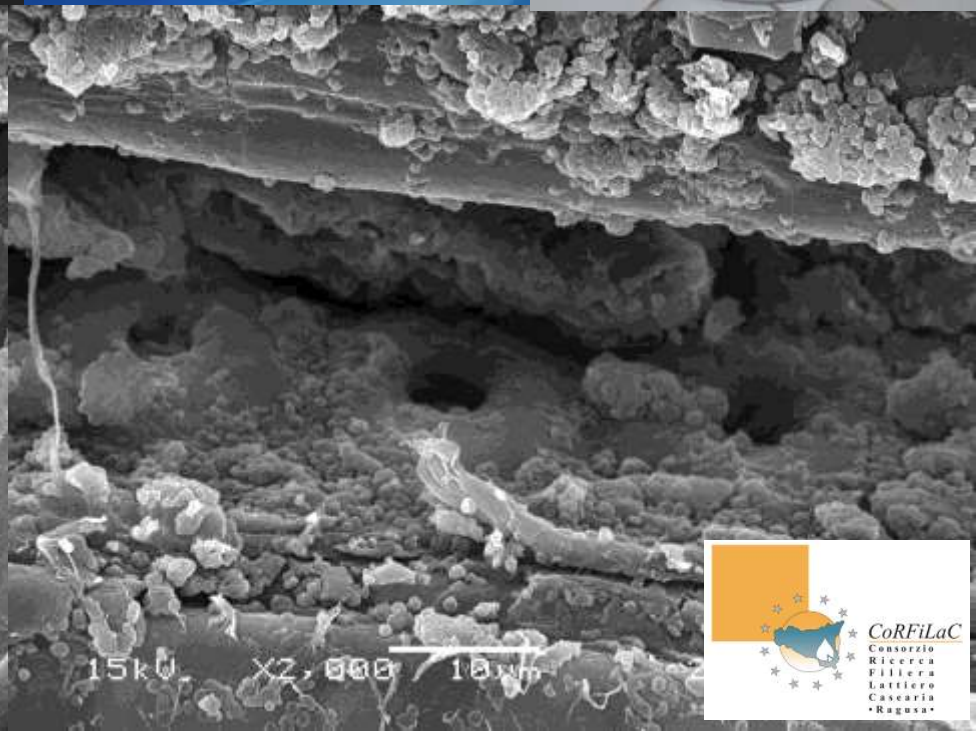
15kV X2,000 10µm
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INTRODUCTION

Although, over 40 different micro organisms were identified and all contributing to the final aroma composition of the cheese, the production of lactic acid can be mainly contributed to the natural milk flora and to the "tina" biofilms that are released in milk when they are brought in contact (Licitra et al., 2007).

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INTRODUCTION

Tina biofilm and natural milk flora play an important role in the determination of the general aroma components of the product (Carpino et al., 2008). However, the contribution of these two bacterial sources, have not yet been completely investigated.

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INTRODUCTION

Based on the fact that all micro organisms have their own group of enzymes, producing their own range of volatile metabolites (Gardner et al., 1998) during their growth, it can be assumed that each different biofilm tina will be also resulting in the production of different aromatic compounds and thus a different overall aroma profile when inoculated into milk.

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OBJECTIVE

To investigate the development of volatiles' and odor active compounds in relation to microbial growth in milk which has been inoculated with biofilm from different *tinas* (under the usual cheese-making procedure except for the rennet addition step)

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Materials and Methods

In order to investigate the influence of the aroma compounds formed in milk attributed exclusively in the tina biofilms, pasteurized milk was used. This way any interference from aroma compounds caused by the natural micro-flora of raw milk that is usually used in the real manufacture of Ragusano cheese, was avoided.

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Motivation

In addition to that, the selection of such a type of milk was also based on studies that have shown that treatments of milk such sterilization, homogenization or ultra high pressure homogenization (UHPH), may result in the formation of elevated volatile compounds like aldehydes or methyl ketones interfering with the volatiles to be measured (Contarini et al., 1997, Valero et al., 1999; Vazquez-Landaverde et al., 2005; Pereda et al., 2008;)

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Materials and Methods

The tina biofilms used in this study were collected from the inner surface of the wooden vats (500 cm^2) from 11 different farms designated as A - K.

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Materials and Methods

The 11 biofilm populations of A-K farms were initially thawed and later inoculated separately into pasteurized milk samples.

The initial inoculum of each biofilm placed respectively in the milk samples was approximately in the range of 10^4 to 10^5 total cfu of biofilm/ml of milk sample.

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Materials and Methods

The choice of this initial inoculum quantity was based on the fact that from previous studies, it was found that after 10 minutes of contact of micro filtrated milk with these specific tina biofilms (A-K) the total viable cfu was found to be $2*10^5$ cfu/ml on average (also depending on the initial total cfu/ml concentration of the biofilm) (Lortal et al., 2009).

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SAMPLE TREATMENT

BIOFILM SAMPLES
from the inner surfaces of each tina (five different sampling areas of 100 cm^2)

Inoculum in UHT milk:
INOCULATED MILK SAMPLES

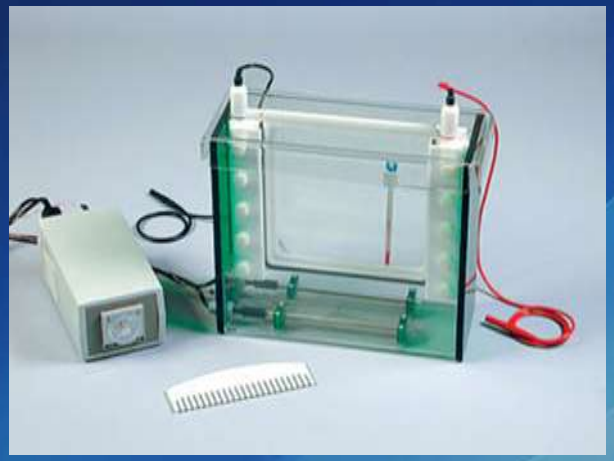
INCUBATED UNDER RAGUSANO CHEESE-MAKING CONDITIONS:

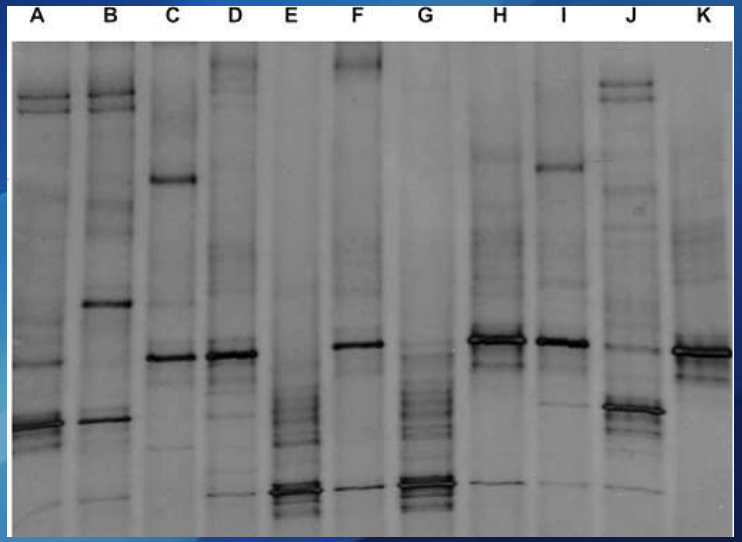
65 min at 35° C
45 min at 40° C (first cooking)
120 min at 45° C (second cooking)
24 h at 15° C (time before brining)

Incubated samples were first analyzed by TTGE-DDGE, bacteria counting and Smart Nose®.

DDGE

PCR-DGGE amplicons of 11 incubated milk samples





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SMARTNOSE, GC/Mass, GCO

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Materials and Methods

Smart Nose analysis was performed, on the 11 biofilms inoculated milk samples, to select the samples which were significantly discriminating VOCs for further analysis:

- **GC/Mass**
- **GCO**

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It is known by literature that micro organisms need first to adapt, grow, and reach a bacterial number in the range of 10^4 to 10^7 depending on the type of bacteria (Haugen et al., 2006; Magana et al., 2001), before the metabolites that they produce can be detected. Therefore, before the samples were analyzed by the Smart nose, the bacterial counting was performed to assure that the incubation time was long enough to allow specific components to be formed and detected.

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MATERIALS AND METHODS

SMart Nose

The first artificial nose of a new generation of instruments based on mass spectrometry

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MATERIALS AND METHODS

Extraction by SPME

(DVB/CAR/PDMS)

5 ml of sample

Water temperature: 40 ° C
Extraction: 30 min
Fiber exposition: 30 min

GC/Mass system: capillary column HP-5

(30 m X 0.25 mm X 0.25 μm film thickness)

MATERIALS AND METHODS

Extraction by SPME

(DVB/CAR/PDMS)

5 ml of sample

Water temperature: 40° C
Extraction: 30min.
Fiber exposition: 30 min.

Gas Chromatography Olfactometry

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MULTIVARIATE ANALYSIS
FULL CROSS VALIDATION

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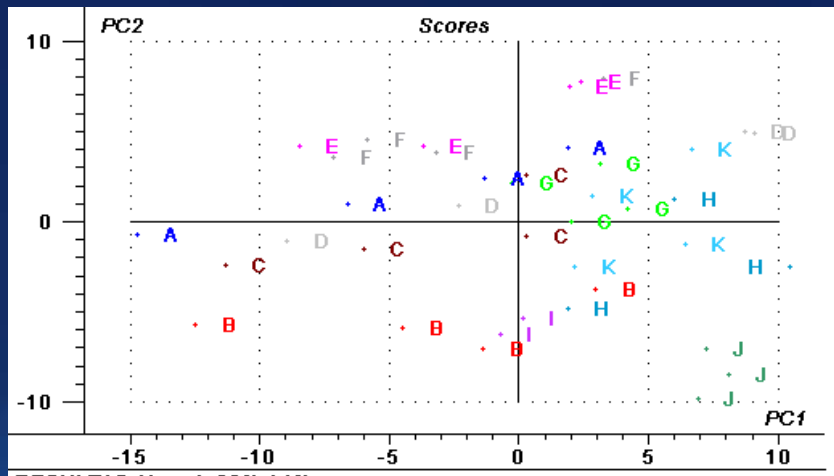
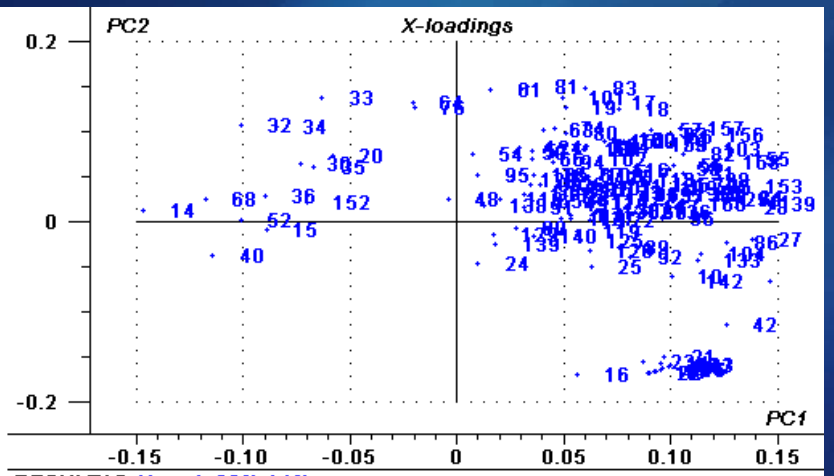
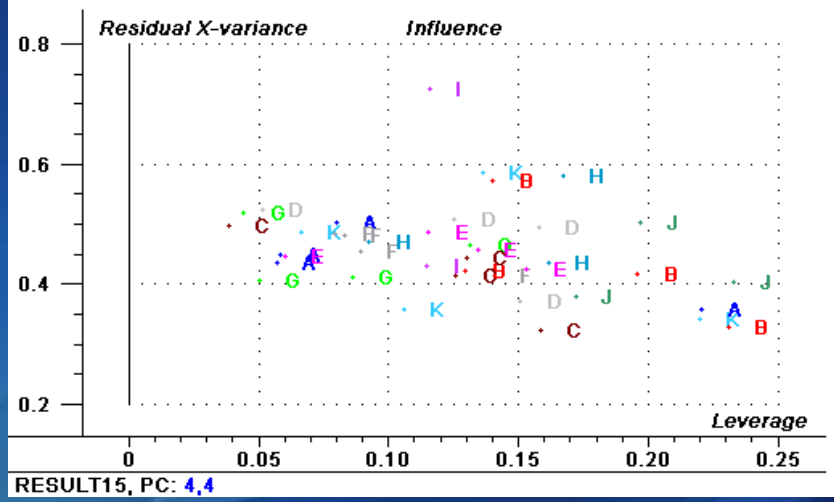
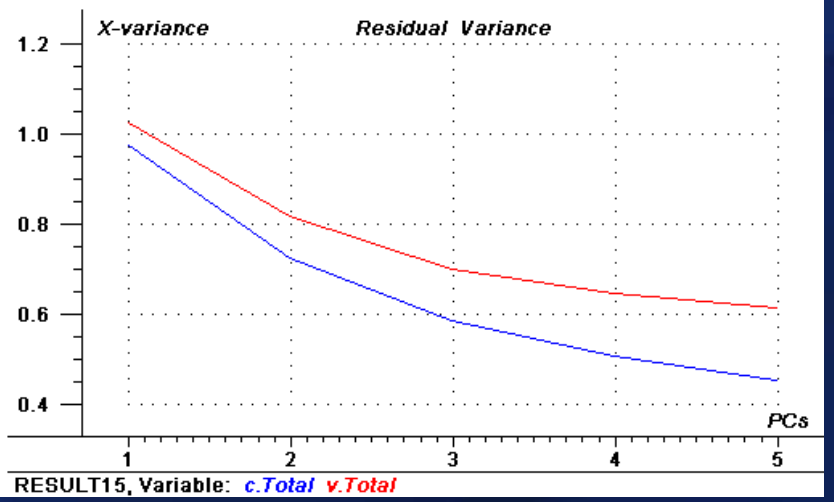
SMARTNOSE

ALL 11 SAMPLES



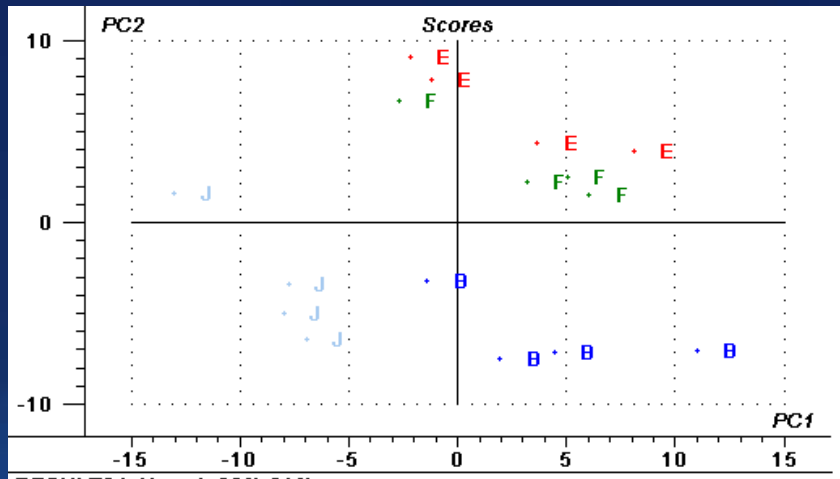


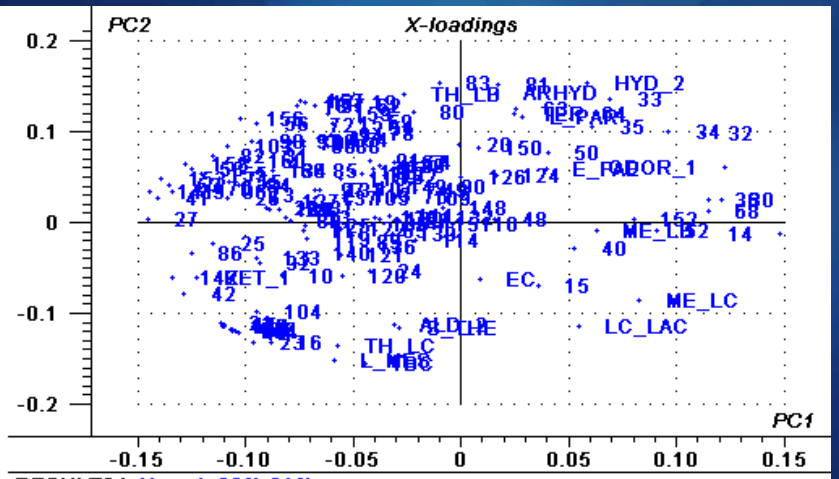


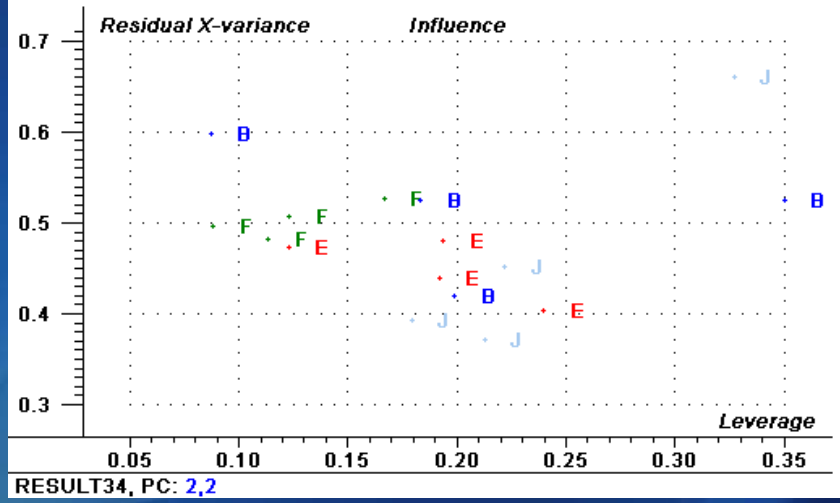


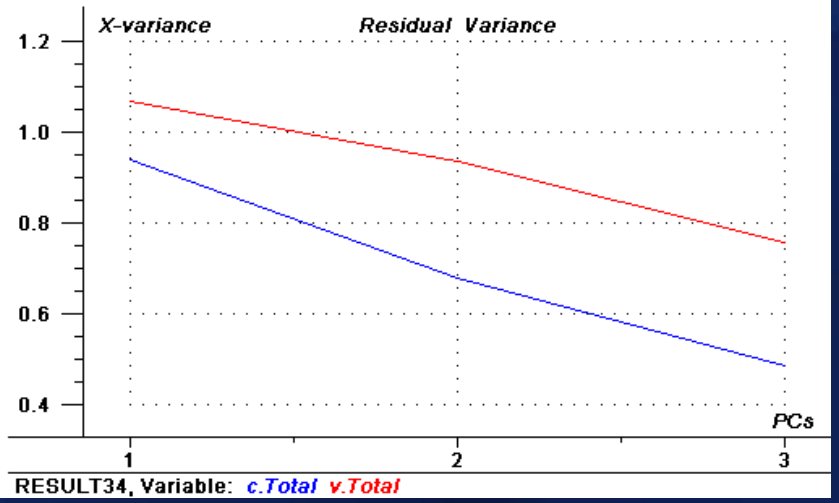
RESULTS FOR THE 4 SELECTED SAMPLES:

SMARTNOSE, TTGE-DDGE, GCO, GC/MASS









Four (B, E, F, J) were selected as they were representing the greatest volatiles' variation (PC1 45%;PC2 25%).

Samples B, E, F, and J were also further analyzed by GC/O and GC/MS

Samples E and F were similar in profile, but different from B and J. Profiles of B and J differed also.

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GENERAL RESULTS

		GCO	
pentanol	apple	1	
(Z)-2-nonenal	hay	1	
2-nonanone	hot milk	1	
methyl thiophene	garlic	1	
2-hexenol	hay	2	
ethyl hexanoate	apple/orange	2	
dimethyl disulfide	garlic	2	
(E)-limonene oxide	green	2	
(E)-2-nonenal	herbaceous	3	
octane	burnt/solvent	3	
methyl geranate	soapy	3	

Odor active compounds which distinguished samples belonged to three groups.

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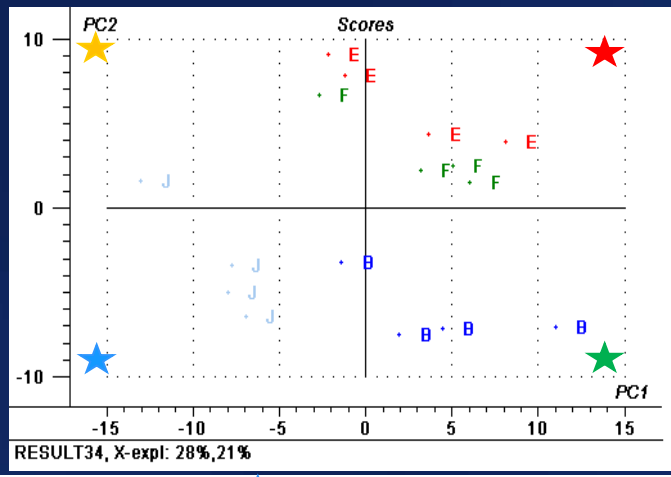
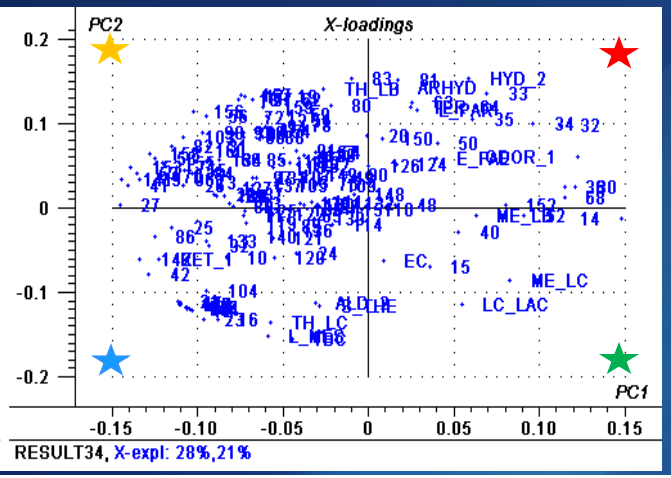




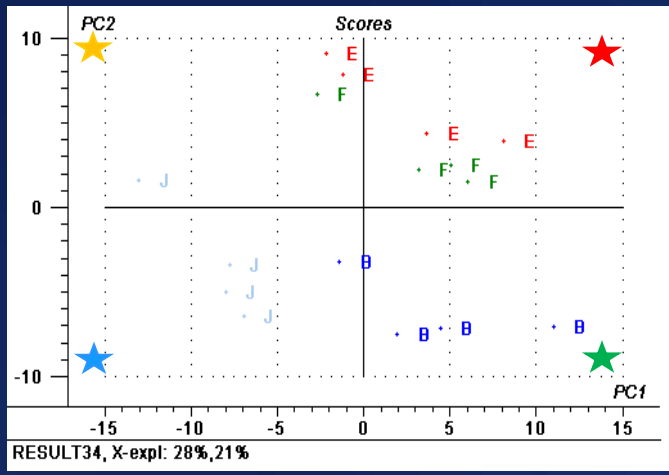
Inversely related

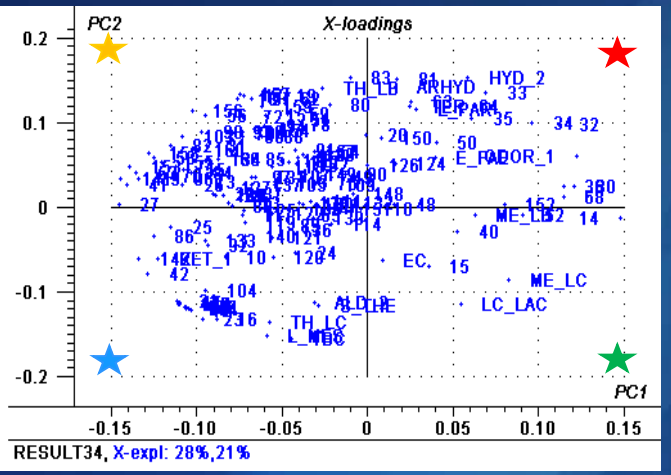
Item (same frequency)	Absence	Presence	Item (same frequency)	Absence	Presence
Lactobacillus Plantarum	E, F	B, J	2,2,4,6,6-Pentamethyl-heptane	B, J	E, F
Leuconostoc Meseneroides	E, F	B, J	Odor group 2	B, J	E, F

Odor group 2:

- 2-Hexenol; hay note
- Ethyl-hexanoate; apple orange note
- Dimethyl-disulfide; garlic note
- (E)-Limonene-oxide; “green” note

The 1st group distinguished samples E, F from samples B, J by the presence or absence of L. Plantarum and Ln. Meseneroides at the same time and negative relation to 2,2,4,6,6-Pentamethyl- heptane and odor complex 2.



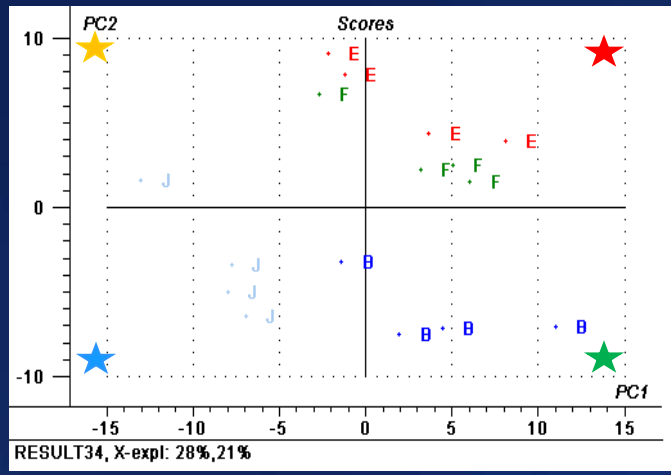


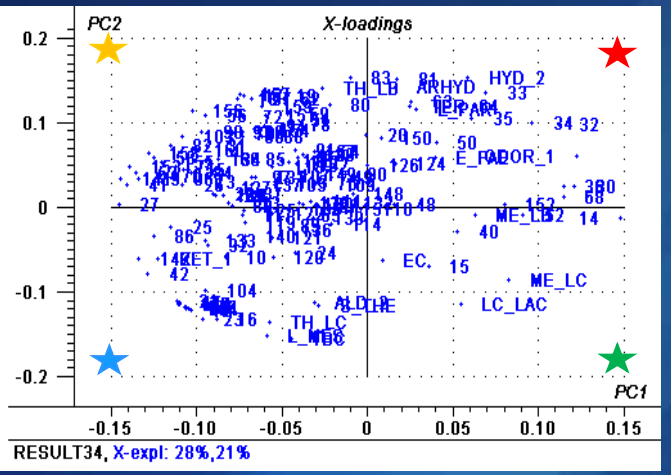
Item (same frequency)	Absence	Presence
Lact. Helveticus	E, F, J	B
Lc. Lactis	E, F, J	B
1-Octanol	E, F, J	B
Odor group 3	E, F, J	B

Odor group 3:

- (E)-2-Nonenal; herbaceous note
- Octane; burnt/solvent note
- Dimethyl-disulfide; garlic note
- Methyl-geranate; soapy note

The 2nd group distinguish sample B from samples F, B, J by the presence or absence of Lactobacillus Helveticus, Lactococcus Lactis, 1-octanol and odor groups 3 at the same time.





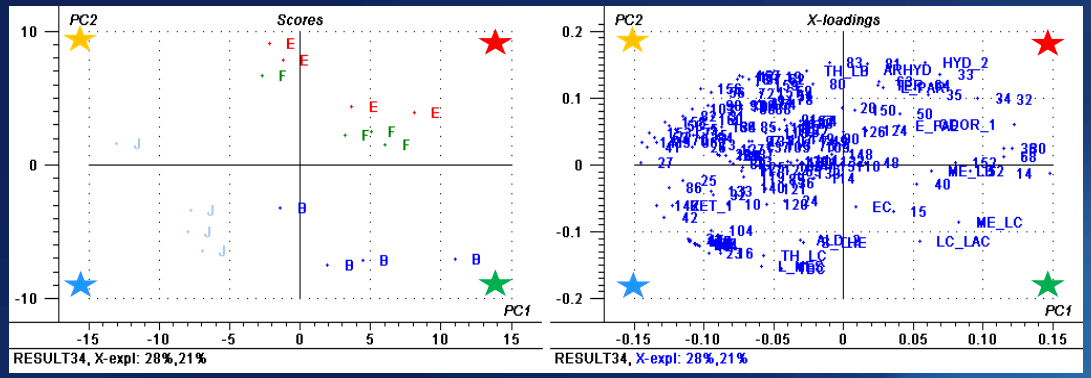
Inversely related

Item	Absence	Presence	Item	Absence	Presence
2-Dodecanone	J	B, E, F	Odor group 1	B, E, F	J

Odor group 1:

- Pentanol; apple note
- (Z)-2-Nonenal; hay note
- 2-Nonanone; hot milk note
- Methyl-thiophene; garlic note

The 3th group distinguished sample J from samples E, F, B by the presence or absence of either 2-dodecanone or odor group 1.



★ ⟵ **Inversely related** ⟶ ★

Lactobacillus Paracasei = 2,3,4-Trimethyl-hexane

Streptococcus Thermophilus = Octanal

★ ⟵ **Inversely related** ⟶ ★

Lactobacillus Delbrueckii = 1-Methyl-3-(1-methylethyl)-benzene

Nonanoic acid =
1-Pentanol =
3-Methyl-butanal =
Tridecanal =
2-Undecanone =
2,4,6-Trimethyl-octane

= means equal frequencies

CONCLUSIONS

Incubated milk samples differed in volatiles' composition.

These differences were associated to microbial composition and to Tina biofilm, as a consequence.

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CONCLUSIONS

- **It was evident that *Lactobacillus plantarum, Leuconostoc meseneroides, Lactobacillus Helveticus,* and *Lactococcus Lactis* were most responsible for the separation of volatile composition of the incubated milk samples.**

- **Most of the investigated bacteria were associated to specific odor and volatiles' compounds.**

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CONCLUSIONS

Each tina biofilm had a different behaviour regarding aroma releasing when inoculated into milk under certain conditions;

Individual bacteria were associated to distinct volatile compounds.

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Grazie per la vostra attenzione !!!!!!!!!!!!!!!!!