UNIVERSITÀ DEGLI STUDI DI MILANO

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BMMIB – Behind Microbe-Microbe Interactions in Blue Cheese

[AGR 16]

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Index

1.Introduction

1.1. Historical and Technical Hints

Cheesemaking have started about 8000 years ago and now there are more than 1000 cheese varieties worldwide (Sandine and Elliker, 1970), each with unique flavour and form. Blue cheese represents a peculiar example of cheese whose production relay heavily on the balance between microorganisms consortia and environmental conditions. The Blue cheeses are mainly characterised by the use of the mould *Penicillium requeforti* (Cocolin *et al.,* 2009) during the production process, which confers them a typical appearance and flavour. Many countries have developed their own types of Blue cheese, each with different characteristics and manufacturing methods. The best-known varieties today, worldwide, are considered to be Gorgonzola, Roquefort, Stilton and Danablu, all of which have been granted the status of Protected Designation of Origin/Protected Geographical Indication (PDO/PGI).

Gorgonzola is the most famous Italian blue cheese; its tradition is very long in Italy and the first evidences of Gorgonzola production date back to 879 A.C. The story tells that in the small town of Gorgonzola, which is built in the exact direction from which the shepherds came down from the mountains in the direction of Milan, this cheese was born casually (Savini, 1936), obtained from the mixing of two types of curds, the cold one from the evening, with the hot one in the morning – here the definition of “two-pastes-Gorgonzola”, not in use anymore. After the spontaneous acidification of raw milk, this primitive cheese was posed in natural caves in the Valsassina Mountains or in ripening-intended structures built *in loco* (Cornabba, 1912); here, thanks to the precarious hygienical conditions and to the mix of cold and hot curds that created caves inside the cheese paste, it was naturally colonized by indigenous *Penicillium roqueforti* populations.

In the subsequent years productions technology improved continuously and in the XII century first evidences of a primitive “inoculum” came out: in fact, cheese makers introduced the use of a coagulant, the calf rennet, that was previously get mouldy until it became green, to help the insemination of spores and make the final product as more homogeneous as possible. After the first insemination Gorgonzola wheels were posed in the “purgatory”, a 22°C environment in which they lost serum and they dry completely, in order to proceed to their salting and piercing: this last passage is fundamental to allow the oxygen to enter in the wheels and to start germination of *P. roqueforti* spores into the vegetative form (Trieu-Cuot and Gripon, 1983). The ripening of this primitive version of Gorgonzola continued then in the already cited natural caves or huts, and was very long, from three to five months.

As time passed, the overwhelming needs of the consumers and the contemporary discovery of bacterial cultures, in particular of Lactic Acid Bacteria (LAB) at the end of XIX century, brought an air of change in Gorgonzola production, and the introduction of specific Lactic Acid Bacteria selected cultures started to become normal in the second post war period. However, formerly in the beginning of XX century, in the *Regia Stazione Sperimentale Del Caseificio* in Lodi, first experiments with selected *P. roqueforti* spores suspensions were carried on (Samarani, 1910). It is credit of professor Renko of *Centro Sperimentale del Latte (CSL)* the introduction of this new method with unique curd and the use of selected LAB and *P. roqueforti* cultures. He tested several LAB species, finding out that two species used for yogurt production, *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, were the most adaptable ones (Renko and Ghitti, 1958). The introduction of these selected thermophilic cultures allowed to standardize the product and limiting defects. In 1996, Gorgonzola was awarded with the Protected

Designation of Origin (PDO) from the European Commission (Commission Regulation No 1107/96).

1.2. Technology of Modern Gorgonzola Productions

Nowadays, raw milk have been replaced with pasteurized milk: this fast operation, that typically lasts for 40/55 seconds at 72°C, guarantees the lighting of milk's natural microbial charge, especially the psychrophilic one, that, otherwise, would develop at low temperatures, which are typical of Gorgonzola maturation, and would compete with the other components of the microflora, conditioning the final product characteristics (Ghitti *et al.*, 1995). The introduction of the "unique paste" technology introduced the problem about the creation of the internal caves, where the inoculated *P. roqueforti* spores could naturally develop, could be achieved. For this reason modern Gorgonzola is always inoculated, along with thermophilic LAB and mould spores, with yeasts – typically *Saccharomyces cerevisiae* – that have the technological function of inflating the wheels and creating the internal space for the mould development, with the carbon dioxide produced in a fermentative way (Florèz and Mayo, 2006): the carbon source for this kind of metabolism is still not clear. After the adding of rennet and an initial acidification by LAB, the curd is then cut into pieces of the size of a hazelnut and placed in cylindrical plastic bands called "Fascere", to form the cheese wheels. During this stage – not longer than one day and with a temperature of 20-22°C and 90% humidity – the curd is posed onto cloth sheets that help the operator to turn cheeses upside down several times for whey drainage without pressing [**Figure 1**]

COW MILK

↓

PASTEURIZATION (72 °C per 40 sec)

↓

COOLING OF MILK TO 30 °C

↓

INOCULATION WITH NATURAL STARTERS

Streptococcus termophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Saccharomyces cerevisiae

↓

INOCULATION WITH *PENICILLIUM ROQUEFORTI* SPORES

↓

ADDING OF LIQUID RENNET (approx. 50 ml/100 Kg)

↓

CLOT BREAKING (curd size approx. 2.0 - 2.5 cm)

↓

REMOVING CLOT AND POSING IN "FASCERE"

↓

RETAINING THE CLOT (10 h at 18°C) WITH 4 TURN OVERS OF THE WHEELS

↓

YEAST DEVELOPMENT STAGE IN CABIN/"PURGATORY" (48h at 22°C)

WITH SALTING OF WHEELS (with a saturated solution of NaCl)

↓

4-6°C RIPENING FOR 55-70 DAYS WITH PIERCINGS OF WHEELS AFTER ONE AND TWO WEEKS

With 85-90% humidity

Figure 1: Flowsheet of sweet Gorgonzola production.

Gorgonzola possesses physical and chemical properties that lead to a selection on the growth and diffusion of specific yeasts such as high fat (23-30% w/w) and proteins (19-24% w/w) concentration, high carbon sources amount, significant salt concentration and the presence of citric acid and acetic acid. In addition, it is generally stored at temperatures lower than 10°C (Gobbetti *et al*, 1997). Usually, among the yeasts species involved in the production of Gorgonzola there is *Debaryomyces hansenii*, present only on the rind of cheese thanks to its best tolerance to NaCl, while *S. cerevisiae* is present both inside and on the surface of the cheese (Roostita and Fleet, 1996). After posing the cheese in the Fascere, between the second and the fourth day of production, the wheels are leaved inside a 22°C cabin to inflate and lose the water in excess. After that phase, wheels are moved to the cold rooms at 4-6°C and 95% humidity, where the real Gorgonzola ripening starts. During this longer passage, wheels are pierced two times, a week away from each other and salted (Lomonaco *et al.*, 2009). *Penicillium roqueforti* veins normally appear in the centre of the cheese after between 22 and 36 days, while spores formation by the mould is usually completed after 49 days (Gobbetti *et al.,* 1997). The introduction of this entire selected microflora allows a very much shorter ripening period, decreasing it to 60-90 days, thanks to the accelerated acidification and proteolysis of the cheese, which is faster to reach the correct characteristics for selling.

The production of blue-veined cheeses, including Gorgonzola, involves a maturation stage that is characterized by the growth of a complex ecology of yeasts, bacteria and filamentous fungi. Microflora development and matrix formation play a pivotal role in the development of cheese characteristics. The microbiological interactions and associated biochemical activities that occur during this stage determine product acceptability and value through their impacts on sensory quality, shelf life and safety. In particular, the ability of pathogens to grow in cheese is

largely dependent on the manufacturing steps required for cheese making (extent of acidification by the starter culture, the amount of heat applied at various stages during the manufacture, ripening/maturation conditions), the physicochemical characteristics of the cheese (pH, salt content, water activity), and the growth requirements of the microorganism (Cheeseman *et al.,* 2014).

The knowledge about the interactions between bacteria, yeasts and fungi has been poorly investigated. Gorgonzola production followed few rules, but was overall leaved to the scroll of natural events. Also nowadays, the amounts of LAB, *S. cerevisiae* and *P. roqueforti* starter cultures do not follow scientific rules but they are often blended and adjusted on the basis of the evolution of the previous fermentation process, especially in minor production plants. Since every microorganism contribute to the quality of the final product adding the aroma, taste and texture, it is mandatory to investigate these interactions in all the cheese sections in order to improve the flavour and the ripening of the cheese.

1.3. *Penicillium roqueforti*: the main actor in Gorgonzola production

As mentioned previously, *Penicillium roqueforti* is the mold that, developing in the cheese paste, gives it the characteristic grain with shades that shimmer from green-blue to glaucous green to dark green. For this reason, cheeses that permit the natural development of this filamentous fungus are called blue cheeses, and in particular in Italy they are named “Erborinati” – from “Erborin”, the dialectal name for parsley in Lombardy. It was firstly isolated and characterized from another famous blue cheese from which it took its name, the French production Roquefort (Raper and Thom, 1949). Within the varieties, there are different biotypes

with different physiological characteristics, which differentiate the cheese maturation and mostly the appearance and flavor of the final product, so we can have the following type of blue cheeses (Ghitti *et al.*, 1995):

- “Very spicy”: Roquefort, Danablu, Edelpilzkäse;
- “Moderately spicy”: Bleu d’Auvergne, Bleu de Bresse, French Blue cheeses generally, Stilton, Spicy Gorgonzola;
- “Sweet creamy paste”: Sweet Gorgonzola, Saingorlon, Fourme d’Ambert

Under the taxonomical profile, the species *Penicillium roqueforti* Thom has been distinguished and described for cheese production in different groups (Salvadori *et al.*, 1962): the strain used for the production of the “Sweet Gorgonzola” – which is nowadays the most famous and consumed one in Italy – belongs to the *N* group, which collects the strains used for all the “Sweet creamy paste” blue cheeses production (**Table 1**). It is a group characterized by a pale green appearance on plates and a low proteolytic activity, in comparison with other groups, as reported by previous works on milk caseins degradation (Bianchi Salvadori, 1987). The other variety of Gorgonzola, called “spicy” or “natural”, is characterized by a harder texture and a more pungent flavour, given both by a *P. roqueforti* strain belonging to the *Nat* or the *P* group, which is featured with a stronger proteolytic and lipolytic activity and a darker green appearance, along with a major acidification in the first production phases, with a consequent massive loss of water.

Strain	*Use in dairy industry*	*Proteolytic activity*	*Lypolitic activity*
N group	Sweet creamy paste blue cheeses, like Sweet Gorgonzola, Saingorlon, Fourme d'Ambert	+	+
P group	Spicy Gorgonzola, Edelpilze, Bleu de Bresse, Danablu	++	+++
B group	Very spicy German and Danish blue cheeses	+++	++++
Nat group	Isolated for the first time by Savadori in 1974, used for the Spicy Gorgonzola production in recent times	++	++

Table 1: strains within the *Penicillium roqueforti* Thom species, classified by Savadori and colleagues (1962) based on their application in dairy industry. Proteolytic activity has been measured by the detection of tyrosine freed in the medium by milk-cultivated spores; lipolytic activity has been measured by the detection of by-products of oleic acid degradation, like azelaic and nonanoic acids.

As well as for the proteolytic activity, *Penicillium roqueforti* is a fundamental component of Gorgonzola for its meaningful contribution to the organoleptic and sensorial properties that make this cheese unique. The mould is not the only actor in the formation of fragrant or tasty compounds: for example, *Saccharomyces cerevisiae* is able to produce diacetyl starting from pyruvate in presence of Coenzyme A (Chuang and

Collins, 1968), and this compound is known to be a perfect descriptor of buttery flavour (Collins, 1972).

However, the contribution of the mould to the overall picture of sensorial components is considerably higher, due to its lipolytic activity against triglycerides and fatty acids that frees a large amount of flavour- and odour-related compounds. In fact, this fungus owns two types of lipases, an alkaline and an acid type that attack fatty acids via a β-oxidation reaction (Hwang *et al.*, 1976); this reaction, along with secondary reductive reactions, releases a large quantity of volatile compounds, like alcohols, ketones esters and free fatty acids. Those compounds are linked to a series of sensorial descriptors that could concern both odour or flavour of the cheese: regarding odour, for example, the carboxylic acid butanoic acid or the ketone 2-heptanone are fundamental for the typical putrid scent that characterize blue cheeses, the alcohol 2-heptanone is linked to an oily odour, while the ketones 2-pentanone or 2-nonanone are typical for their fruity and cheesy combined smell, which is a main characteristic especially in the Sweet variety of Gorgonzola (Moio *et al.*, 1999). All the Gorgonzola varieties described present this volatile compounds profile, but the different membership of *Penicillium roqueforti* strains, used for the two types of production, to different taxonomical groups, could modify the proportions in which these compounds are present, changing in this way the sensorial profile, along with the texture, of the final products, that are significantly different one from each other.

1.4. Detection of *Penicillium roqueforti* in cheese matrix

Since *P. roqueforti* is the main actor in Gorgonzola cheese ripening, for cheese manufacturers the growth control of fungal populations is crucial to ensure that cheeses are properly and rapidly colonized, which avoids the implantation of contaminants (Nielsen *et al.*, 1998), but mostly to guarantee the correct manufacturing of a product that always has to withstand to the severe regulations of the PDO certification.
It was previously reported (Gobbetti *et al.*, 1997) that the enumeration of *P.roqueforti* colonies could be performed by classical microbiological methods, as dilution and plating on proper medium like Yeast Glucose Chloramphenicol; with methods like this, however, fungal hyphae cannot be quantified, because mostly spores, rather than fungal mycelium, usually grow on plates (Le Dréan *et al.*, 2010). Since on food particles the mold is usually present in a filamentous hyphal condition and the number of countable spores is higher than the real biomass production – due to the presence of conidia that produce large numbers of spores – the need of an alternative quantification method for fungal biomass is clear.
While methods like optical microscopy analysis or dry weight assessment are already outdated, due to their scarce relevance in an industrial context (Taniwaki *et al.*, 2006), indirect biochemical methods are still in use to quantify fungal biomass especially for assessment of presence of spoiling molds in cereals (Dong *et al.*, 2006) and soils (Montgomery *et al.*, 2000). These methods are mainly based on ergosterol quantification, which however is extremely variable between fungal strains, different growth conditions, age of the population: this aspect, together with the required lipid extraction – that could further reduce the sensitivity of the method in lipid-rich substrates like ripened cheeses – make this a questionable method for industrial continuative applications.

In the last years the Polymerase Chain Reaction (PCR) has make inroads as a quantitative and qualitative method for detection of prokaryotic or eukaryotic microorganisms in food samples, as this technique permits to associate a quantity of DNA to the relative presence of the target species on which primer sets have been designed (Niessen, 2008). Specific qRT-PCR have been recently used for assessment of fungal presence in red-smear cheeses (Larpin *et al.*, 2006), and has proven to be a simple and reproducible method for precise measurements, based on the fact that a single spore is related to a single DNA molecule: if the copy number of the target gene is known, the amount of DNA found could be related to the quantity of spores present.
However, when the goal is the quantification of fungal biomass considered as mycelium, this approach results more complex, since mycelium is a multinucleated-cells organism: this particular feature makes impossible the association between DNA quantity and fungi biomass, whereas it is not correct the association between DNA and number of cells, as it possible to do with spores. As previously considered in soils analysis (Guidot *et al.*, 2002; Landeweert *et al.*, 2003), expressing the results as mycelial DNA amount per gram of substrate may be sufficient for a relative comparison of fungal abundance, especially in cheese samples.

As reported previously, proteolysis level in a Gorgonzola product is another very important index of the cheese quality: in the sweet variety, the more the proteolysis advance, the more creamy and high qualitative the product will be. While a very small part proteolysis in this cheese is due to LAB action, the major part of this maturation process is caused by *P. roqueforti* strong enzymatic activity, as previously reported (Kinsella and Hwang, 1976). For this reason, this activity could be another interesting indicator of correct fungal development in the product. Techniques like

mass spectrometry are well known and highly precise for the characterization of proteolysis profiles in blue cheeses (Gillot *et al.*, 2017), but they have also some problems, like scarce cost-effectiveness in an industrial view, and difficult treatments requested for samples preparation, especially in a complex matrix like cheese. In previous studies, a recombinant *Streptococcus thermophilus* biosensor sensitive to glutamic acid and peptides containing glutamic acid was described (Arioli *et al.*, 2007). Assuming that *P. roqueforti* proteolytic activity is able to increase glutamic acid and or peptides containing glutamic acids in the cheese matrix, the *S. thermophilus* biosensor could represent a rapid and cost-effective method to follow in real time, during cheese production, the proteolysis which is directly linked to the final quality of the product.

2. Objectives of the Thesis

The main aim of this research project has been the description of the microbial interactions occurring during the production process of a sweet PDO Gorgonzola cheese. To this aim, the microbial populations involved in cheese production were monitored based on standard microbiological methods together with the development of molecular and biochemical methods. To reach a complete characterization of the microbial dynamics a metabolomic approach was also applied at each stage of cheese production and ripening. Moreover, since microbiological interactions are pivotal for the features of the final product, LAB-yeasts-*Penicillium* interactions has been studied *in vitro* and *in vivo*, with particular attention to yeast activity in the first production phases. A detailed experimental design of this project is presented below.

Part 1: Microbial population dynamics in sweet Gorgonzola cheese by metataxonomic and classical microbiological approaches

Microbial development and matrix formation play a pivotal role in the development of cheese characteristics. Indeed, the production of blue-veined cheeses, including Gorgonzola, involves a maturation stage that is characterized by the growth of a complex ecology of yeasts, bacteria and filamentous fungi. The microbiological interactions and associated biochemical activities that occur during this stage determine product acceptability and value through their impacts on sensory quality, shelf life and safety. The information obtained in this part of the research project should allow us to have global understanding about microflora composition, dynamics and evolution in order to optimise process conditions and also promote the growth of desirable species. The first part of the project has been focused on setting up the experimental approaches starting from the selection of samples. These samples were provided by Fratelli Oioli cheese-maker (Cavaglietto, Novara, Italy).

Metataxonomics. The growth profiles of individual species throughout the time frame of production will be estimated by metataxonomic analysis. To

characterize microbial population, the total DNA will be extracted from the different matrixes, as will be described in detail in **Chapter 3**, and taxonomic composition of the microbial communities based on *16S rRNA* gene profiling for the bacteria populations and ITS profiling for fungi (Wolfe *et al.*, 2014) will be performed. To identify the different species of microorganisms present in the sample, V4 region of *16S rRNA* (for bacteria identification) and ITS (for yeast identification) will be amplified using a broad-range, specific primer pairs and sequenced.

Microbiological approach. The microbiota characterization and the study of dynamics of microbial communities will be also monitored using culture-dependent methods. These methods allowed us to follow the development of the starter culture strains (*S. thermophilus*, *L. delbrueckii*, *P. roqueforti* and *S. cerevisiae*) and contaminants during all the steps of cheese production.

Part 2: Development of a microbial biosensor to monitor the ripening process in sweet Gorgonzola PDO cheese

In order to address the biochemical and physical changes inside the cheese matrix during the ripening, we will apply and optimize a completely novel method for the detection of glutamate and glutamate-containing peptides in cheese extracts coming from different productions or phases of the same production. We hypothesized that the proteolysis that *P. roqueforti* operates on the cheese matrix during ripening could set glutamate and glutamate-rich peptides free in the aqueous medium. The aim of this workplan, with the glutamate-sensitive recombinant *S. thermophilus* MIM 945 (Arioli *et al.*, 2007), was the monitoring of the proteolytic activity associated to *P. roqueforti* growth. The final aim was to setup an assay useful as a real marker for the correct product development and ripening. The quantification of free glutamate will be carried out indirectly by the measurement of the light amount produced by the recombinant strain, as described in detail in **Chapter 4**.

Part 3: Development of a species-specific RT-qPCR assay for the quantification of *P. roqueforti*

To define the quantitative relationship between microbiological and biochemical changes during ripening, each component of the cheese microbiota needs to be quantified, including the mycelium (hyphae) of fungal populations such as *P. roqueforti*. Therefore, the aim of this task is to develop a qPCR method for the quantification of *P. roqueforti*. In recent years, the Polymerase Chain Reaction (PCR) method has been used successfully to detect and identify fungi. In food mycology, application of PCR-based methods for identification of filamentous fungi has been extensively developed notably for mycotoxin producing species (Niessen, 2008). The qPCR method will be tested directly on cheese samples collected in different stages of ripening, and will be discussed in detail in **Chapter 5**.

Part 4: Evolution of microbial population dynamics in sweet Gorgonzola cheese and identification of the critical control production step that can affect the final cheese quality.

The molecular tools developed will be applied to identify the "critical control production steps" that could have a key role on the final quality of the product. **Chapter 6** will present the final work of this project. To this aim, two different productions will be monitored coupling the classical microbiological approaches with the molecular quantification of *P. roqueforti*, the evaluation of cheese ripening through the use of *S. thermophilus* biosensor together with: i) the quantification of milk sugars and microbial metabolites, ii) the analysis of nitrogen fractions, iii) the quantification of lipolysis by GC-SPME-MS analysis. Low molecular weight peptides, detectable in the 600 kDa nitrogen fractions range, as well as chetones and organic acids derived from fatty acids degradation are of pivotal importance for the flavour and odour panel of this

cheese (Moio *et al.*, 1999; Smit *et al.*, 2005), and fundamental for the final product quality, that has to respect the severe restriction of its PDO origin certificate.

3. Microbial population dynamics in sweet Gorgonzola PDO cheese by ITS and *16S rRNA* gene profiling

3.1. Evolution of Gorgonzola cheese microbial communities using a metataxonomic approach: *16S rRNA* gene profiling

The evolution of microbial communities during Gorgonzola cheese production was carried out using a metataxonomic approach based on *16S rRNA* gene and ITS profiling to address respectively bacteria and yeast populations.

In the two Gorgonzola cheese productions, the microbial communities that characterized each production steps, showed relevant differences thus underlining difficulties in the process standardization. The bacterial starter culture (*S. thermophilus* and *L. delbrueckii*) which have been added to the pasteurized milk, were detected to be dominant starting from the cabin or after the second piercing. Surprisingly a high relative abundance of *S. thermophilus* (36%) was detected in raw milk of 2015 production thus indicting a possible contamination source in the milk pipe line. On the other hand, in 2016 production (**Figure 2b)** *Lactobacillus* DNA was the most represented, with a 47,07% of relative abundance, while *Streptococcus* DNA was almost absent (0,33%). Moreover, in 2015 row milk a high relative abundance of *Micrococcales* was observed (**Figure 2a**), and in specific the species *Cellulosimicrobium cellulans* which was also detected in the latter phases of the ripening. In 2016 production samples, the *Cellulosimicrobium* genus was detectable only in the raw milk at high relative abundance. *Cellulosimicrobium* genus has been already described as a natural milk and cow's intestinal trait contaminant (Gobbetti *et al.*, 1999), but its persistence, especially in the rind in the latter ripening phases in 2015 samples is still a matter of investigation, even because at 20 days of ripening the relative abundance of this taxon reaches 78,01% of the total bacterial DNA. Another interesting difference between the two productions is represented by the sample at 12 hours in cabin (12Ca): in fact, while in 2015 samples the DNA composition of the bacterial population was mainly represented by *Streptococcus* and *Lactobacillus* genera, respectively at 47,36% and 48,11% of relative abundance, with a

minor presence of the previously described *Micrococcales* member, in 2016 samples (**Figure 2b**), a completely different population composition was observed. In particular, in that case, the most represented genus was *Paenisporosarcina*, with the 64,99% of the total DNA, with a lower presence of *Lactobacillus* DNA (16,31%) and an extremely low presence of *Streptococcus* (1,10%). In this sample the presence of members of *Bifidobacterium* and *Bacillus* genera members was also significant, but the major component of the total DNA, *Paenisporosarcina*, drew our attention: in particular it is a genus of psychrophilic bacteria, most of them recently isolated from ice sediments (Reddy *et al.*, 2013). We hypothesized, that the high abundance of this genus could represent the evidence of a contamination in the cooling systems inside the production plant. It is still not clear why in 2016 sample this kind of contamination was observable.

The biodiversity of the microbiota can be analysed through several different methods, the most commonly used being the alpha and beta diversity indexes. Alpha- and beta-diversity have been therefore evaluated on the metataxonomic data of 2015 and 2016 cheese samples. Concerning the alpha diversity, two indexes are used: CHAO1 and Simpson index. The CHAO1 is a measure of richness intra-sample that reported the number of OTUs per sample. More OTUs are present in a sample more the value of CHAO1 is high. Simpson index is another way to measure the alpha diversity. This diversity index depends not only on OTUs richness but also on the evenness, or equitability, with which individuals are distributed among the different OTUs. With Simpson index 0 represents infinite diversity and 1, no diversity. The beta diversity indexes measure the biodiversity by evaluating the similarities between different group of samples. The bacterial profiling defines each sample in analysis. Each sample, which is characterized by a large range of variables derived from the bacterial profiling, is compared to the other samples, creating a distance matrix based on the grade of dissimilarities. The final results of the beta diversity are represented in a PCA graph where, conventionally, the

samples are plotted in multidimensional Euclidean space. The more the points in the graph are close to each other, the more the profiling are similar

The results obtained for *16S rRNA* gene profiling evidenced significant differences between 2015 and 2016 samples based on alpha-diversity using both CHAO1 and Simpson index, as showed in **Figure 3**. The alpha-diversity data highlighted a highest diversity in 2015 cheese samples considering milk, rind and paste. The beta-diversity data underlined further the microbial community differences between 2015 and 2016 cheese samples. In 2015 cheese samples (**Figure 3b**) rind samples grouped together and they were well separated from paste samples underlining high differences between the microbial communities of the two cheese micro-environments. On the other hand, these differences in the microbiota composition of rind and paste samples were not detectable in 2016 cheese samples.

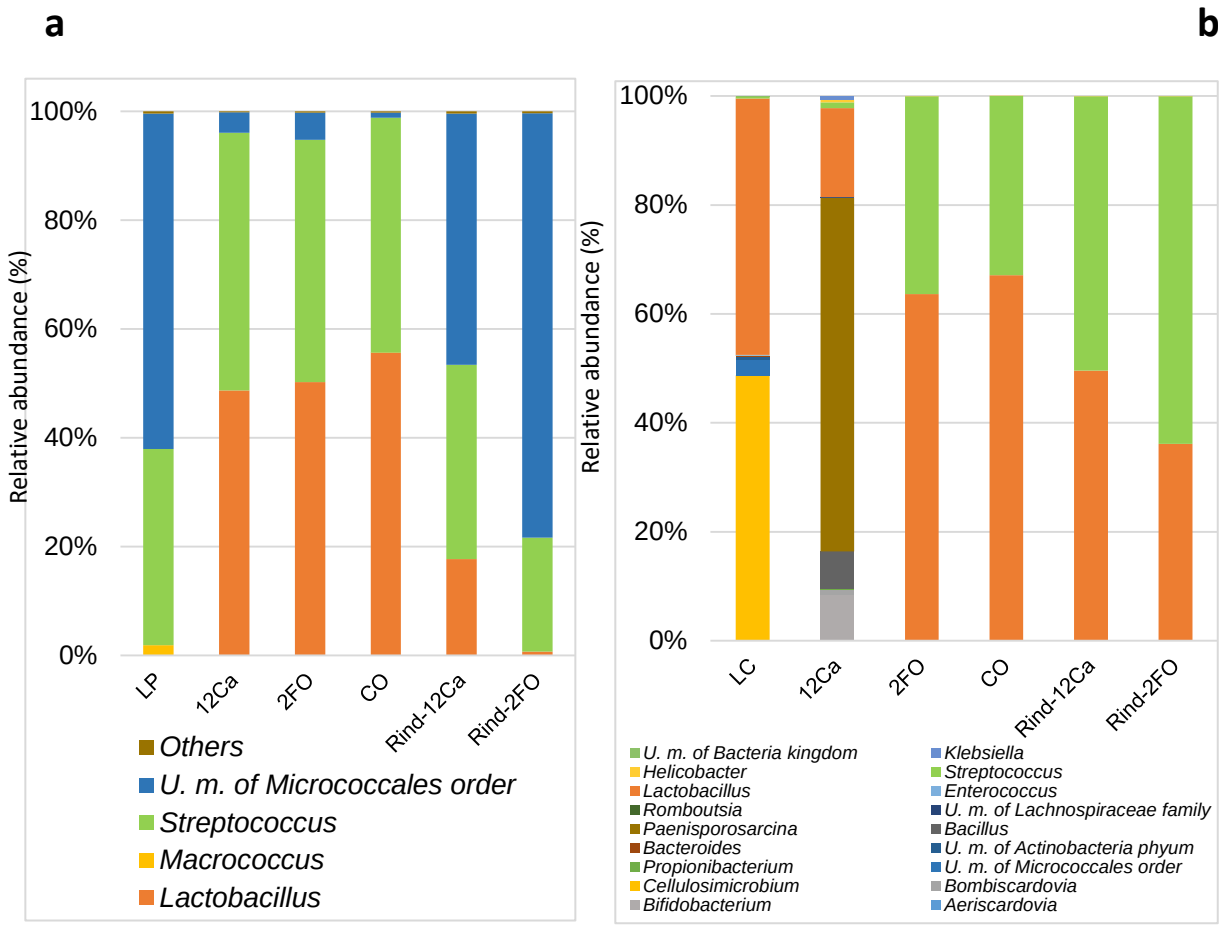


Figure 2: Metataxonomics, represented at genus level, as calculated from *16S rRNA* gene profiling. Only taxa with relative abundance > 1% are represented. **a)**, 2015 samples; **b)**, 2016 samples. **LC**, Crude milk; **12Ca**, after 12 hours in cabin; **2FO**, after the second piercing; **CO**, after 37 days of ripening, **RIND-12Ca**, Rind after 12 hours in cabin, **RIND-2FO**, Rind after the second piercing.

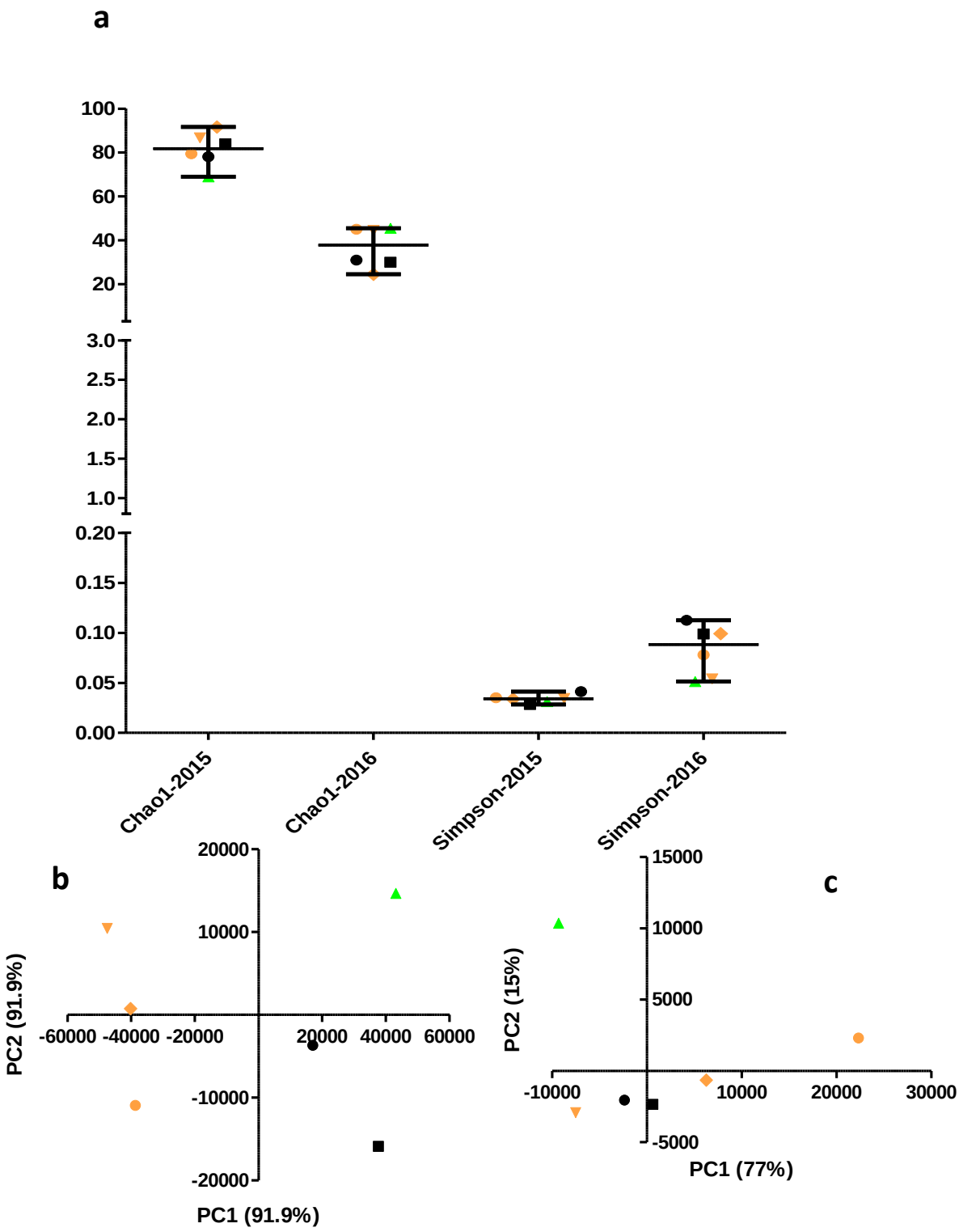

a
100
80
60
40
20
3.0
2.5
2.0
1.5
1.0
0.20
0.15
0.10
0.05
0.00
Chao1-2015
Chao1-2016
Simpson-2015
Simpson-2016
b
20000
10000
-10000
-20000
-60000 -40000 -20000
20000 40000 60000
PC2 (91.9%)
PC1 (91.9%)
c
15000
10000
5000
-5000
-10000
10000
20000
30000
PC2 (15%)
PC1 (77%)

Figure 3: **a)** Alpha diversity for *16S rRNA* gene profiling in 2015 and 2016 samples using chao1 and simpson indexes. **b,c)** Beta diversity for ITS profiling in 2015 (**b**) and 2016 (**c**) samples; ▲, pasteurized milk; ●, Day 1 + 12 hrs in cabin; ●, rind after 12 hours in cabin; ▼, after second piercing; ■, rind after second piercing; ◆, after 37 days of ripening.

3.2. Evolution of Gorgonzola cheese microbial communities using a metataxonomic approach: ITS profiling

The ITS profiling on eukaryotes genera, showed substantial differences between the two productions as well (**Figure 4**). As for the *16s rRNA* analysis, also in that case 2015 samples presented a simpler profile, with only 10 genera represented, in comparison with 24 genera represented in 2016 samples. The first data which attracted the attention was the strong presence of *Debaryomyces* genus in both the productions, especially on rinds in the latter phases (89.36% in the 2015 production, 94,94% in the 2016 production); although its presence as a natural contaminant of dairy productions was already reported (Gobbetti *et al.,* 1999) it was interesting to note that plate counts data of 2016 samples on YGC medium confirmed its presence overcoming the abundance of *Saccharomyces cerevisiae*, especially on rinds (**Figure 6**). Unfortunately, in 2015 *Debaryomyces* species have not been counted on plates. Regarding *Saccharomyces cerevisiae*, there was some discrepancy between the plate counts and the metataxonomic data: in particular, while in 2015 production *S. cerevisiae* DNA was detected (**Figure 4a**), with a peak of 98,36% in 12Ca samples, in 2016 samples *S. cerevisiae* showed a 1,33% of relative abundance in 12Ca samples (**Figure 4b**). This last data was not in agreement with plate counts, where, instead, the yeast showed a concentration comparable to that measured in the previous production (9,2 x 10^6 CFU/g 2015, 6,4 x 10^6 CFU/g 2016). It was hypothesized that higher

numbers of *Debaryomyces* species had biased the relative amount of *S. cerevisiae* DNA during the analysis. Another interesting data was related to the presence and relative abundance of the principal actor of Gorgonzola ripening, *Penicillium roqueforti* which was detected with high relative abundance (74,27%) in 2016 production, thus indicating a possible problem in the metataxonomic analysis. Finally, while the fungal population in 2015 samples was dominated, together with *Saccharomyces* and *Debaryomyces* genera, by an unknown member of *Dipodascaceae* family (with a peak of 22,76% of relative abundance in 12Ca samples from rinds) in 2016 samples this population is more underestimated, in favour of *Candida* genus that reached 51,77% of total fungal DNA in samples collected after the second piercing (2FO).

. Unlike what previously observed on *16S rRNA* gene profiling, the alpha-diversity measured on ITS profiling metataxonomic data did not reveal significant differences between 2015 and 2016 cheese samples using both CHAO1 and Simpson indexes. The high diversity of ITS sequences could justify the high values of alfa-diversity measured (**Figure 5a**). Nevertheless, despite a comparable alpha-diversity, 2015 and 2016 samples showed remarkable differences in beta-diversity thus confirming the differences in eukaryotes taxa detected in 2015 and 2016 cheese samples respectively. In addition, as a consequence of the presence of *P. roqueforti*, *S. cerevisiae*, *Debaryomyces* and other genera both the rind and in paste samples, the beta-diversity did not allow to group samples based on the cheese microenvironment, as previously observed for the beta-diversity obtained from *16S rRNA* gene profiling **(Figure 3b).**

Based on the data collected from the metataxonomic approach, the main conclusion can be that the complex composition of the microbial and fungal populations inhabiting Gorgonzola during its production phases, is not fully comparable to the cultivable microorganisms counted on plates; furthermore, the big differences found between 2015 and 2016 productions, could be influenced by the working environment. All these data suggested that a metataxonomic approach was not suitable to have a clarification of the total populations' amount, creating the need of a more precise and less environmental conditions-dependent method to unravel the microbial dynamics

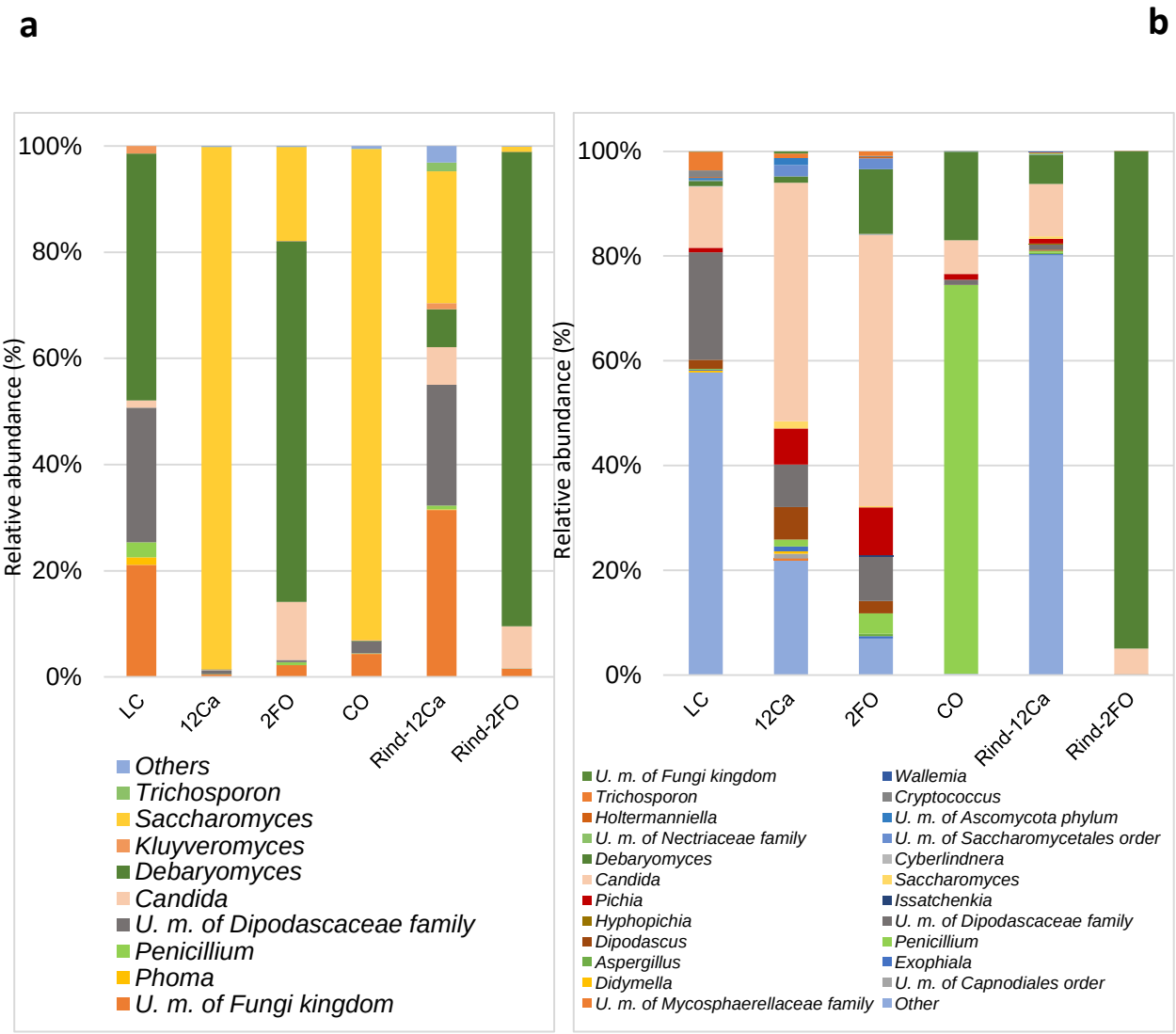


Figure 4: Metataxonomics, represented at genus level, as calculated from ITS profiling. Only taxa with relative abundance > 1% are represented. **a)**, 2015 samples; **b)**, 2016 samples. **LC**, Crude milk; **12Ca**, after 12 hours in cabin; **2FO**, after the second piercing; **CO**, after 37 days of ripening, **RIND-12Ca**, Rind after 12 hours in cabin, **RIND-2FO**, Rind after the second piercing

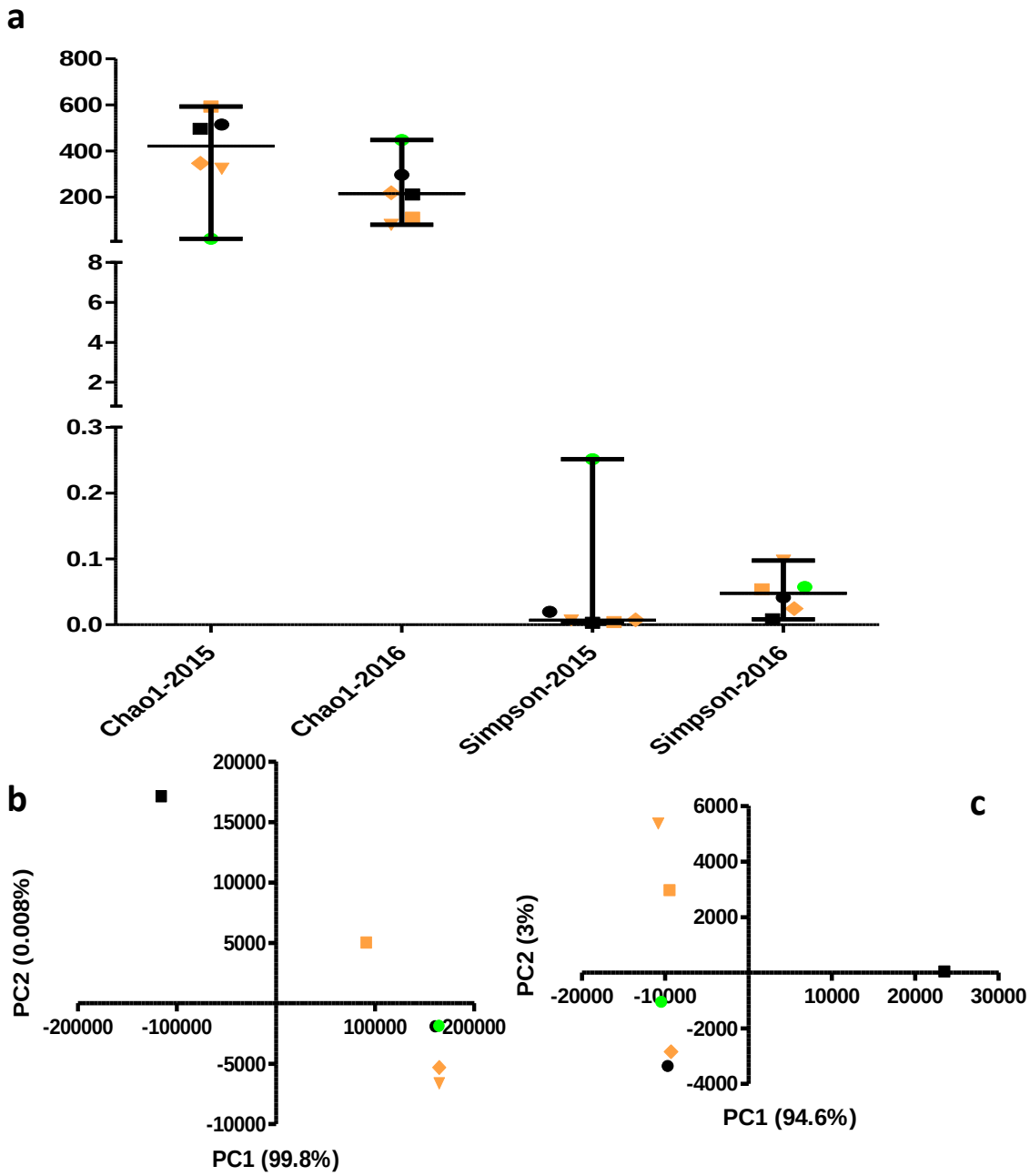


Figure 5: **a)** Alpha diversity for ITS profiling in 2015 and 2016 samples using chao1 and simpson index. **b,c)** Beta diversity for ITS profiling in 2015 (**b**) and 2016 (**c**) samples; ●, pasteurized milk; ▼, Day 1 + 12 hrs in cabin; ●, rind after 12 hours in cabin; ■, after second piercing; ■, rind after second piercing; ◆, after 37 days of ripening.

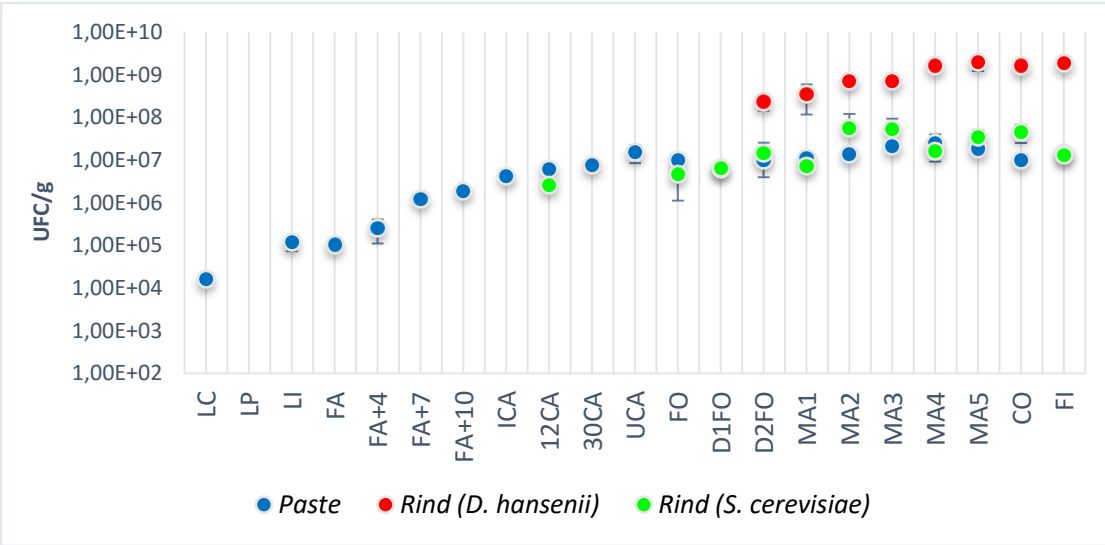


Figure 6: viable counts of yeasts on YGC medium in 2016 samples. Each point is the result of two counts made on two wheels from the same production ± SD. **LC**, Crude Milk; **LP**, Pasteurized Milk, **LI**, Inoculated Milk; **FA**, Fascera; **FA+4**, Fascera + 4 hours; **FA+7**, Fascera + 7 hours; **FA+10**, Fascera + 10 hours; **ICA**, entry in cabin; **12CA**, 12 hours in cabin; **30CA**, 30 hours in cabin; UCA, exit from cabin; **FO**, before first piercing; **D1FO**, 1 week after first piercing; **D2FO**; 1 week after second piercing; **MA1**, 23 days; **MA2**, 26 days; **MA3**, 29 days; **MA4**, 32 days; **MA5**, 35 days; **CO**, 37 days; **FI**, 60 days.

4. Development of a microbial biosensor to monitor the ripening process in sweet Gorgonzola PDO cheese

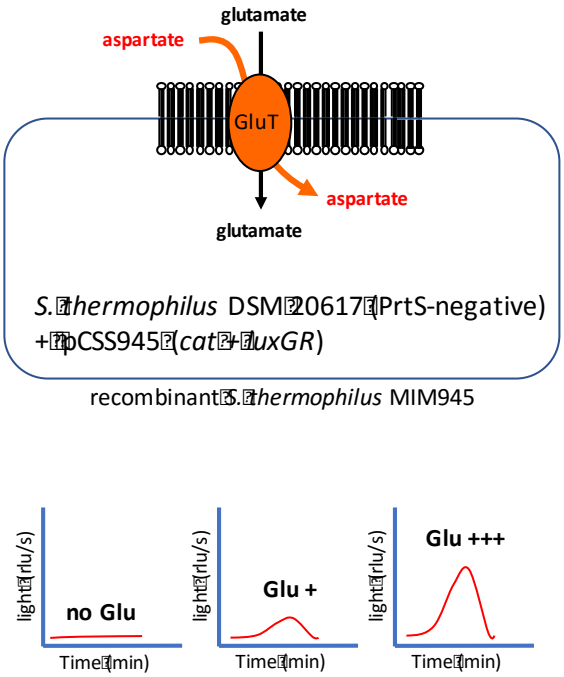
4.1. Introduction

In the production of Gorgonzola cheese, the ripening process associated to the growth of *P. roqueforti* is fundamental to reach the final quality of the product. Even if the ripening process could be considered the consequence of several enzymatic activities associated to rennet, starter and non-starter LAB the role of *Penicillium roqueforti* enzymatic activities is considered dominant (Kinsella & Hwang, 1976). Both lipolysis and proteolysis associated to *P. roqueforti* growth are key factors to reach the sensorial parameters that characterize Gorgonzola cheese and that should be reached to adhere to the severe regulations imposed by the PDO origin denomination (Ghitti *et al.*, 1997). The highest activity of the mould concurs in a pivotal way in the final product soft texture, by the degradation of β- and γ-caseins (De Noni, 2008), lowering the concentration of pheylalanine and tyrosine rich peptides, that are well known as bitter peptides that could negatively affect the final product taste. The measurement of proteolytic activity could be therefore a way of major importance for a cheese-producer to follow the ripening process, but techniques like mass-spectrometry or NMR could be very costly, and samples preparation is often labourios and time consuming due to the complexity of the cheese matrix (Gillot et al., 2017). For this reasons there is the need for a new method for the evaluation of proteolysis in real time during cheese production. Here we evaluated the performance of a glutamate sensitive bioluminescent strain of *Streptococcus thermophilus* to address the level of cheese proteolysis on the basis of the amount of glutamic acid in the cheese matrix during the ripening process.

4.2. Results and discussion

4.2.1. Development of a glutamate sensitive biosensor for the quantification of glutamic acid

In previous studies, *S. thermophilus* growth stress induced by defined concentration of aspartic acid and its ability to restore metabolic activity in presence of Glutamic acid was reported (Arioli *et al.*, 2007). Glutamate is very important for cell metabolism, as it can be used in trans-aminase reactions to provide amine-groups to α-ketoglutaric acid from Krebs cycle and rebuilt glutamic acid, with leucine as intermediate, or to restore α-ketoglutaric acid in high energy-demanding phases, via the enzyme glutamate dehydrogenase. As depicted in **Figure 7**, the presence of glutamate can restore the metabolic activity of *S. thermophilus*, and in that way the production of ATP can restart.



1 Recombinant *S. thermophilus* MIM945 was induced in Glu/nitrogen starvation exposing cells to a critical Asp concentration. Asp competes with Glu on the same membrane transporter.

2 Starved cells produced light by a *luxGR* system when Glu is available, *i.e.* exposing cell to cheese extract reach in peptides or free amino acids.

3 The amount of light produced is related to the Glu concentration to which the cells were exposed.

Figure 7: Schematic representation of *Streptococcus thermophilus* MIM945 glutamate-dependent recover of activity and light production.

Here we used a previously described recombinant strain of *S. thermophilus* MIM945 (Arioli *et al.*, 2010), derived from strain DSM 20617 but carrying the plasmid pCSS945, with a complete luciferase operon. The presence of this operon induced the strain to produce light in presence of D-luciferin using a ATP-dependent reaction. ATP is consumed with production of light during the conversion of D-luciferin in oxyluciferin by luciferase (Day *et al.*, 2004).

Assuming that *P. roqueforti* proteolysis should increase the concentration of glutamic acid during Gorgonzola cheese ripening, this characterized biosensor was chosen to confirm this hypothesis. In order to test if the method was reliable, the *S. thermophilus* cells were pre-incubated as described in Materials and Methods, in presence of 2.0 mM Aspartic acid to induce a stress caused by the lack of Glutamic acid, that blocks the activity of a fundamental membrane amino acids transporter (Hols *et al.*, 2005). The experiment was set up with different Glutamic acid concentrations (0.1, 0.5, 1.0, 2.0 mM), to follow the recovering of microbial metabolic activity with the production of ATP. The aliquots of milk with the bioluminescent strain and 0.2 mM luciferin were mixed with an equal volume of milk, containing the respective glutamic acid concentrations mentioned above. A positive control to follow the growth of *S. thermophilus* MIM945 without the addition of 2.0 mM Aspartic acid was added, and showed the reference light production; a second positive control, with a 0.5 mM concentration of Casaminoacids (Difco), showed a high light production, even higher than the positive control itself, because of the high glutamic acid concentration in this aminoacids mixture. The amount of 2.0 mM glutamic acid proved already to be a sufficient quantity to completely restore the microbial metabolic activity after the starvation period induced by aspartic acid (**Figure 8**), thus the method was validated and the concentration of 2.0 mM glutamic acid was chosen as positive control for the experiments performed with Gorgonzola cheese extract.

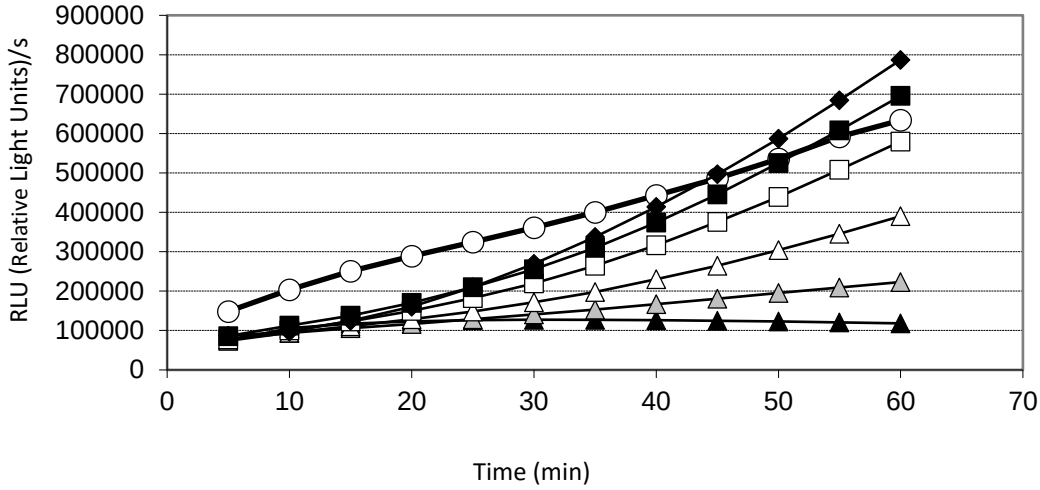


Figure 8: Light emission of *S. thermophilus* MIM945 cells in nitrogen starvation induced by 2.0 mM Asp. Nitrogen starvation was restored by adding Glu at different concentration or Casein hydrolizate. (◯, Light emission of *S. thermophilus* MIM945 in presence of reconstituted skim milk; ▲ , Light emission of *S. thermophilus* MIM945 in nitrogen starvation caused by 2.0 mM Asp; ▲ , Light emission of nitrogen starved *S. thermophilus* MIM945 in presence of 0.1 mM Glu; △, Light emission of nitrogen starved *S. thermophilus* MIM945 in presence of 0.5 mM Glu; □, Light emission of nitrogen starved *S. thermophilus* MIM945 in presence of 1.0 mM Glu; ■, Light emission of nitrogen starved *S. thermophilus* MIM945 in presence of 2.0 mM Glu; ◆, Light emission of nitrogen starved *S. thermophilus* MIM945 in presence of 0.5% Casein hydrolizate).

4.2.2. Use of *S. thermophilus* MIM945 to address proteolysis during Gorgonzola cheese production

Cheese samples collected at different stage of the Gorgonzola production process were homogenized subjected to a double centrifugation step to avoid cheese fat- and protein-rich component, and after that, starved cells of *S. thermophilus* MIM945 were added to these supernatants together with 0.2 mM D-luciferin. The measurement were carried out on a 2015 and a 2016 productions. Light production from *S. thermophilus* MIM945 was monitored and the maximum luminescence reached after a 4 hours exposition was recorded and plotted (**Figure 9**). The results observed showed that between day 20 and day 37, we measured a considerable light emission which was related to the glutamic acid development during cheese proteolysis.

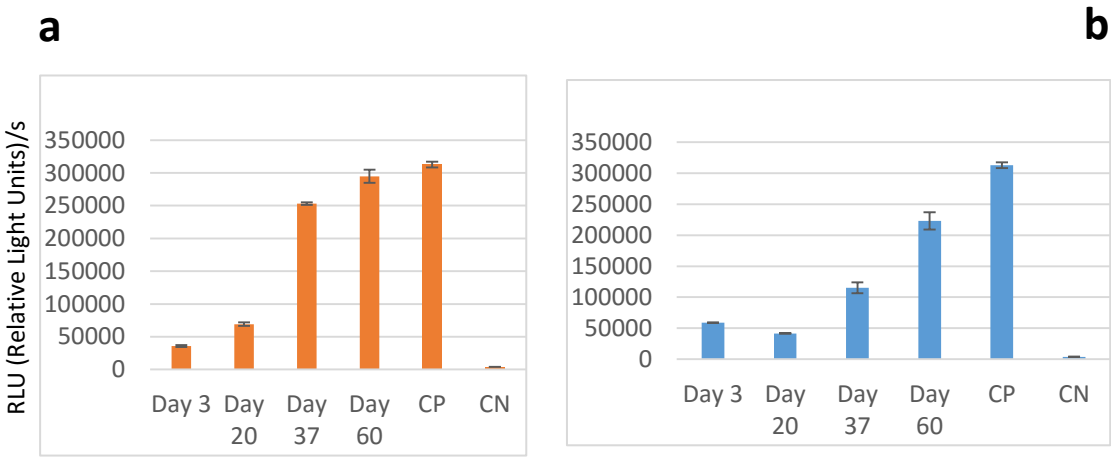


Figure 9: Light emission by *S. themophilus* MIM 945 exposed to Gorgonzola cheese extract. **a)** 2015 and **b)** 2016 cheese samples. Analysis performed only on paste samples. **CP**, nitrogen starved *S.thermophilus* MIM 945 cells exposed to 2 mM Glu; **CN**, nitrogen starved *S. thermophilus* MIM 945 cells without Glu. Data represent the maximum of light units reached by the samples after 240 minutes of analysis. Cheese samples extracts were obtained at day 3, day 20, day 37 and day 60 of production.

4.2.3. Use of *S. thermophilus* MIM945 to address proteolysis in Parmigiano Reggiano cheese with different ripening ages

The *S. thermophilus* MIM945 biosensor was tested on cheese extract obtained from Parmigiano Reggiano, a cheese characterized by a longer ripening process compared to that of Gorgonzola cheese. In a cheese like Parmigiano Reggiano there is no mould that could exercise its proteolytic activity, so the protein consumption is principally due to starter and non-starter cultures, assisted by the long ripening-time. As previously known (Mucchetti *et al.*, 2000), in Grana Padano and other long ripened cheeses, a common characteristic that comes out with cheese age is the conversion of Glutamic acid to Pyroglutamic acid via a thermophilic bacteria-mediated enzymatic process (**Table 2**). As a direct consequence, the glutamic acid concentration should decrease during ripening time. For this reason, a comparison between the behaviour of Parmigiano Reggiano and Gorgonzola cheeses in terms of free glutamic acid was performed; the aim of this comparison was confirming the progressive glutamic acid consumption in long-ripened cheeses, directly validating free glutamic acid as a biomarker in a short-ripened cheese like Gorgonzola . As expected cheese samples collected from 24, 30 and 36 months of ripening showed a light emission which revealed an inverse correlation with the biosensor light emission thus indicating a decrease of glutamic acid during the ripening process in Parmigiano Reggiano cheese (Fig. cc) .

Sample	Cheese	$\overline{X}$	SD	CV	Min	Max	n	D-pGlu (%)	L-pGlu (%)
1 to 18	Grana Padano	504	122.5	24.3	354	722	18	<0.1	>99.9
19 to 30	Bagos	67	41.4	62.0	20	135	12	<0.1	>99.9
31 to 37	Provolone	118	108.5	91.9	11	273	7	<0.1	>99.9
38 to 41	Parmigiano Reggiano	600	134.1	22.3	404	690	4	<0.1	>99.9
42 to 45	Latteria	50	36.8	73.3	16	99	4	<0.1	>99.9
46 to 47	Swiss Emmenthal	131			98	163	2	<0.1	>99.9
48	Pecorino Romano	123					1	<0.1	>99.9
49	Fontina	228					1	<0.1	>99.9
50 to 51	Cooked mountain type-cheese	399			395	404	2	<0.1	>99.9
52 to 53	Gorgonzola	16			11	22	2	<0.1	>99.9
54	Sbrinz	132					1	<0.1	>99.9
55	Edam	17					1	<0.1	>99.9

Table 2: Pyroglutamic acid (pGLU) content in some cheese varieties and evaluation of the relative content of D- and L-pGlu (mg/100 g). (Mucchetti *et al.*, 2000)

Figure 10 depicts the luminometric curves of *S. thermophilus* MIM945 in presence of cheese extracts from different ripened Parmigiano Reggiano cheeses. What can be noticed, is that 36 months Parmigiano Reggiano extracts had a minor recovery capacity on *S. thermophilus* MIM945, due to their progressive conversion of free Glutamic acid to Pyroglutamic acid during the ripening, if compared to a 24 or 30 moths ripened Parmigiano Reggiano. As reported by the authors, this Pyroglutamic acid-conversion activity is not present, or at least present in a very small amount, in Gorgonzola, in other shortly ripened cheeses or in cheeses produced without a thermophilic starter culture. This observation could confirm that in Gorgonzola, mould proteolysis frees a high quantity of Glutamic acid, that, due to the relatively short ripening period and to the strong competition that *P. roqueforti* has towards thermophilic starter cultures, is not converted into Pyroglutamic acid, but remains free in the medium. This situation could be taken in advantage to create a reproducible and precise biosensor, using Glutamic acid as a marker for the correct proteolysis during the ripening period, in a fast and cost-effective way. The comparison proposed in the final chapter of this dissertation will highlight the differences between two

Gorgonzola productions from the same plant in two different years: there the effectiveness of this method could be better appreciated and coupled with other assays to strengthen its scientific goodness.

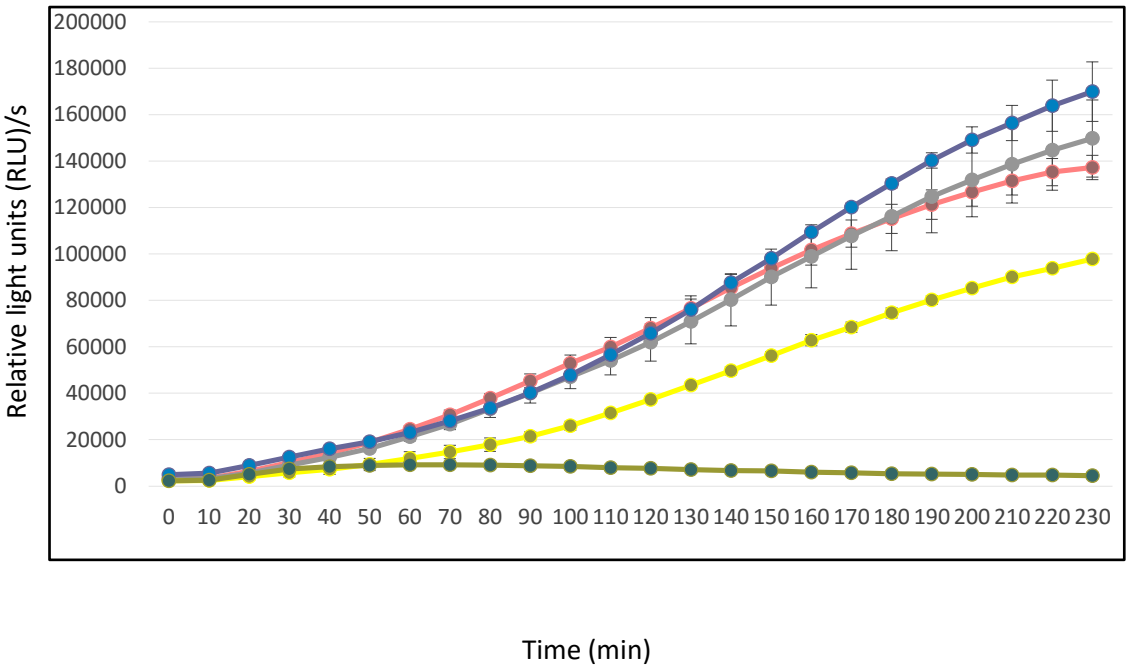


Figure 10: growth curves depicting metabolic activity recovery by *S. thermophilus MIM 945* in presence of Parmigiano Reggiano cheese extracts. (nitrogen starved *S. thermophilus* MIM945 cells exposed to 24 months cheese extract; : nitrogen starved *S. thermophilus* MIM945 cells exposed to 30 months cheese extract; : nitrogen starved *S. thermophilus* MIM945 cells exposed to 36 months cheese extract; : nitrogen starved *S. thermophilus* MIM945 cells exposed to 2.0 mM Glu; :nitrogen starved *S. thermophilus* MIM945 cells without Glu). Growth time: 240 min).

5. Development of qPCR protocol for the monitoring of *P. roqueforti* during the production of sweet Gorgonzola PDO cheese

5.1 Introduction

As previously reported, about the methods of detection of filamentous fungi in food matrixes, there is the need for the development of a fast and cost-effective tool to detect in real time the mould *Penicillium roqueforti* during Gorgonzola ripening, and follow its growth to obtain information about the state of the production. As I already presented most of the methods already used, and all their limits, the only walkable way was the development of a qPCR assay. In recent years, the Polymerase Chain Reaction (PCR) method has been used successfully to detect and identify fungi. In food mycology, application of PCR-based methods for identification of filamentous fungi has been extensively developed notably for mycotoxin producing species (Niessen, 2008). It is an accurate quantitative assay required not only to enforce food labelling procedures and prevent food ingredient contamination, but also for the species-specific detection of *P. roqueforti* among the rest of the fungal population that inhabits Gorgonzola.

For this purpose, a novel primer set was designed. The primer set was targeted to a 223 bp fragment of a biosynthetic gene, named *Ari1*, which encodes a fungal terpene cyclase, aristolochene synthase, involved in the first farnesyl-diphosphate cyclization step that stands at the base of the synthesis of a specific *P. roqueforti* neurotoxin, the PR toxin (Hidalgo *et al.*, 2016). The species-specificity of the set was evaluated and the protocol optimized and tested on 2015 and 2016 productions Gorgonzola samples.

5.2. Results and Discussion

5.2.1. Species-specificity and efficiency evaluation of novel primer set ARI1

The novel primer set obtained, named ARI1, was compared to a previously known primer set for the quantification of *P. roqueforti* mycelium in Camembert cheese, ITSRoq1, designed on a 106 bp region of *18S rRNA* gene of *P. roqueforti*, according to GenBank available sequences of the Interspace Transcribed Region (ITS) of the mold (Le Drèan *et al.*, 2010). In order to demonstrate the major *P. roqueforti* specificity of ARI1 in comparison to ITSRoq1, a qPCR trial was set up with control DNA extracted from *Penicillium chrysogenum* and *Geotrichum candidum* pure cultures. The DNAs were all used in the same amount (5ng/reaction). The results obtained are shown in **Figure 11**.

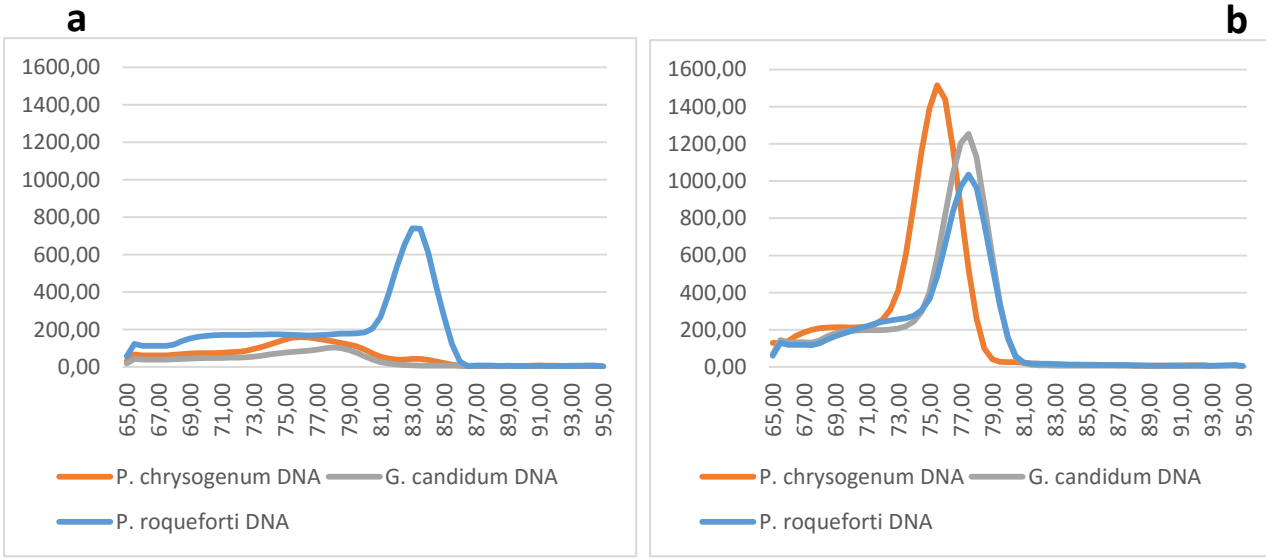


Figure 11: Melting peaks of qPCR fragments depicting the affinity of ARI1 (**a**) and ITSRoq1 (**b**) primer sets for *Penicillium chrysogenum* and *Geotrichum candidum*. Standard curves samples (Std) are not shown.

As it can be noticed, an efficient amplification on *P. chrysogenum and G. candidum* DNA is clear for the primer set ITSRoq1 (Figure xxx/A) but not for the primer set ARI1 (Wells A9, A10, B9 and B10), which is specific exclusively for *P. roqueforti* species. Thus, ARI1 primer set could be used for determinations on Gorgonzola in a quantitative and qualitative way without being affected by the presence of exogenous DNA from species that are very common contaminants of blue cheeses productions, like *G. candidum* (Florez and Mayo, 2006), or from phylogenetically close species like *P. chrysogenum* (Gillot *et al.*, 2015). Once ARI1 primer set was selected for the quantifications on Gorgonzola samples, evaluation of the best annealing temperature to construct a thermal protocol for qPCR reactions was set up. Using a temperature gradient from 50.0 to 58.0°C on a qPCR plate, the best annealing temperature of 50.7°C was obtained: as it can be noticed in **Figure 12**, at the previously mentioned temperature the primer set had the best efficiency **(Fig. 12a)**, the highest melting curve **(Fig. 12b)** and the highest melting peak **(Fig. 12c)**. This annealing temperature was selected to design the thermal protocol for the subsequent experiments.

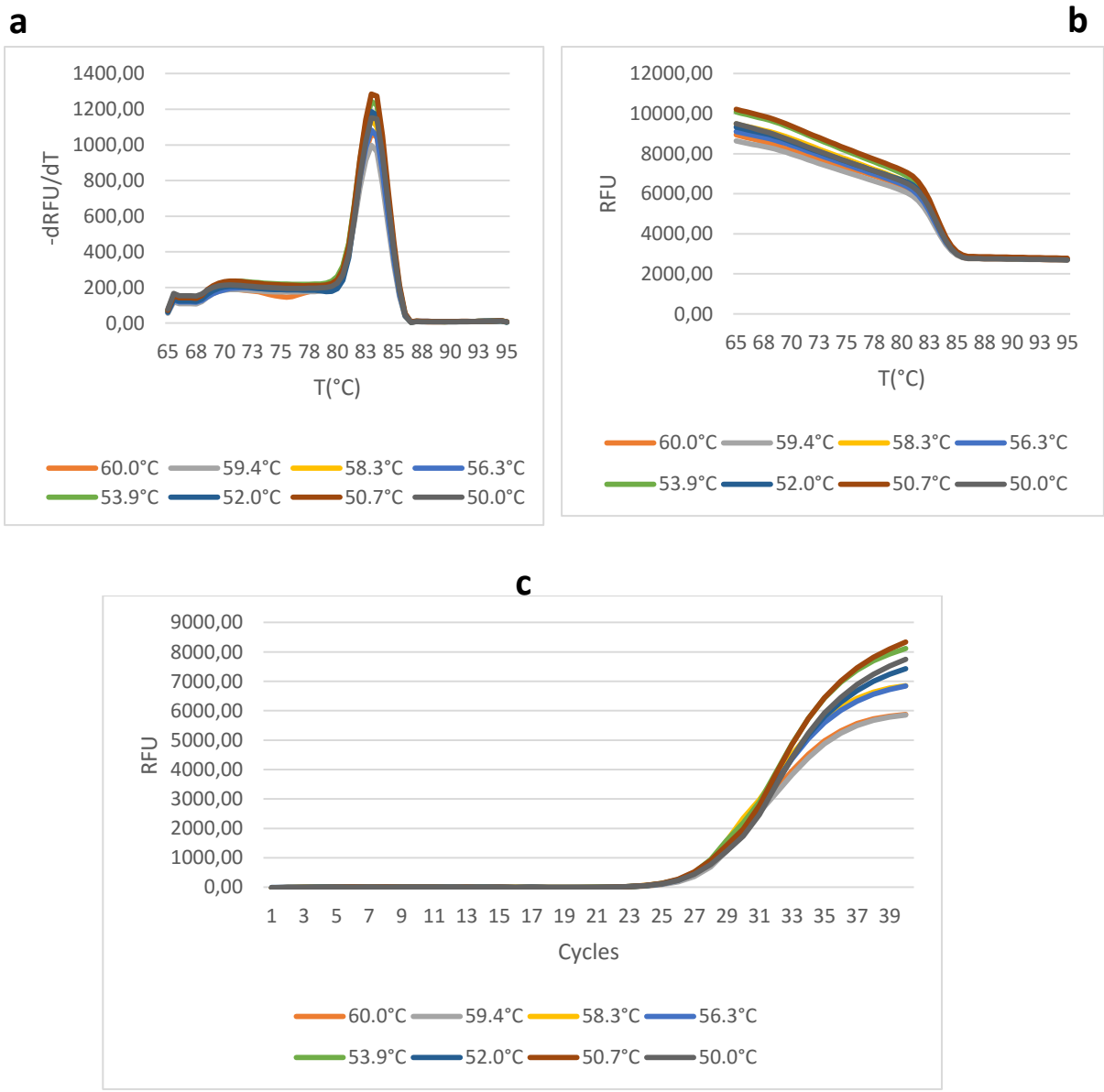


Figure 12: qPCR profiles for the best annealing temperature for Ari1 primer set in a range from 50.0°C to 58.0°C. Efficiency of amplification (**a**), melting curve profiles (**b**) and melting peak profiles (**c**) are reported. The performances of the selected annealing temperature of 50.7°C are depicted in brown colour.

5.2.2. Quantification trials and optimization of a “cleaning” strategy of qPCR products

Quantifications were performed on Gorgonzola samples coming from 2015 and 2016 productions of the same plant. Target DNA was used at a concentration of 5 ng/µl and standard curve was constructed with decimal dilutions of pure *P. roqueforti* DNA extracted from a known amount of mycelium as described in Materials and Methods. What can be quickly noticed in **Figure 13**, is the presence of a key phase in *P. roqueforti* mycelium development: in 2015 production samples DNA extracted from Day 1 to Day 20 (that corresponds to a week after the second piercing of wheels) showed a soiled profile **(Fig. 13a)**, with many non-specific peaks exceeding or under the threshold line, therefore not returning reliable C_t values for the quantification of fungal mycelium. This was observable also for 2016 production samples and an eventual switch to ITSRoq1 primer set did not change the melting profile (data not shown). Curiously, in the period from Day 20 to Day 37 the melting profile of all the samples underwent a complete cleaning **(Fig. 13b)**, giving back clean results from Day 37 until the end of ripening. In 2016 production, a closer sampling in the phase between Day 20 and Day 37 was performed, and what could be observed was a progressive increase in the height of the characteristic *P. roqueforti* 84.0°C melting peak against the detriment of non-specific peaks (Data not shown).

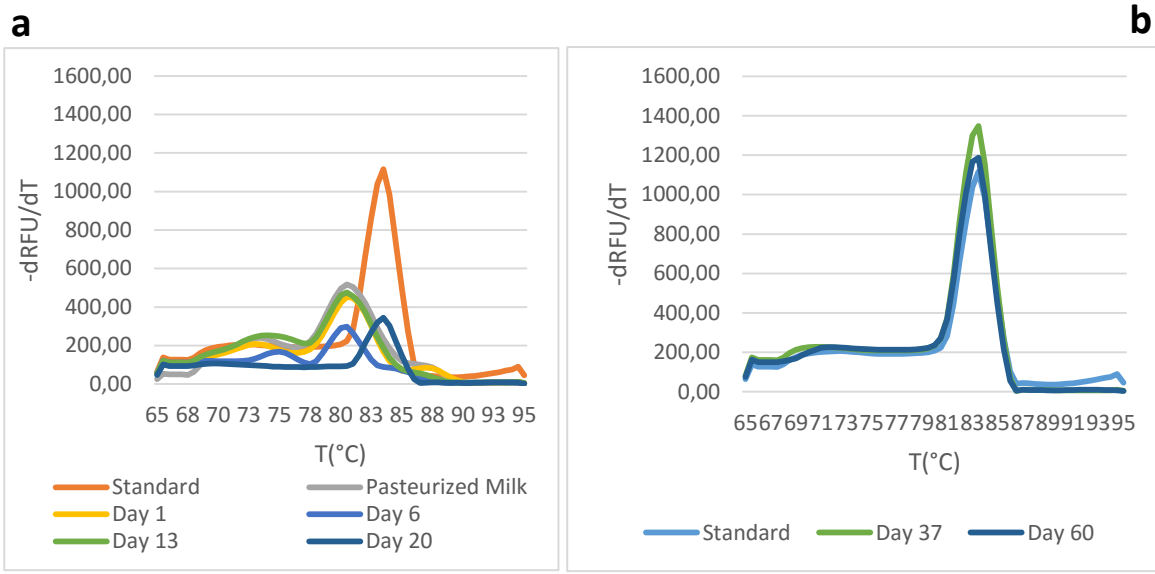


Figure 13: Melting peaks from qPCR quantifications on Gorgonzola samples with Ari1 primer set; **a)** sample profiles from ripening day 1 to day 20; **b)** sample profiles from day 37 to the end of ripening. Samples have been randomly selected in the two phases to highlight the melting profiles.

The hypothesis made was the follow: approximately in the first 20 days the amount of fungal mycelium, and of fungal DNA subsequently, was under the detection limit because of competition with other exogenous DNA present in the sample, while in the subsequent phase the *P. roqueforti* DNA amount became predominant and detectable.

In order to verify this competition hypothesis, a qPCR experiment, with the final purpose of to quantify *P. roqueforti* DNA in the first ripening phase, was set up. For this goal, 5 ng of pure *P. roqueforti* DNA, already used to build the standard curves, was added in reaction to the soiled samples to enrich the presence of the specific DNA, and force the amplification of the fragment. The results are visible in **Figure 14**: as expected, a sample without **(Fig. 14a)** enrichment with pure *P. roqueforti* DNA had a non-specific and non-

quantifiable melting peak, while after the enrichment **(Fig. 14b)** the peak was perfectly detectable. The final quantities were then obtained by subtracting from the numbers, resulted by the interpolation with the standard curve, the known quantity of mycelium correspondent to the 5 ng with which every sample was enriched. The sensitivity of the qPCR method was therefore > 5 ng of *P. roqueforti* DNA. The *P. roqueforti* biomass quantified in randomly selected cheese samples with and without the enrichment step showed differences ranging from 1,08% and 9,93%, and was associated to the intrinsic difference between replicates of the same sample. Applying the quantification in three replicates of the same sample, the variability in the biomass quantified was ranged between 0,98% and 11,21%, thus highlighting a good reproducibility of the method.

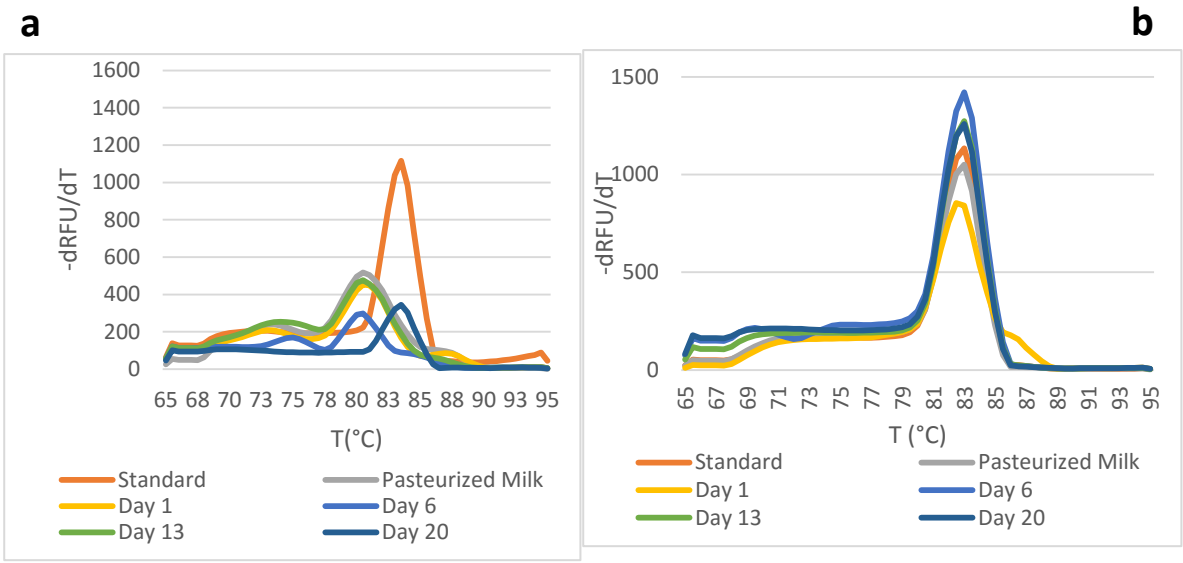


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Figure 14: Comparison between melting profiles of Gorgonzola samples without (**a**) and with (**b**) 5 ng of pure exogenous *P. roqueforti* DNA added in reaction. The same samples before and after the cleaning attempt are highlighted.

This qPCR protocol developed here allowed finally to identify a marker phase of Gorgonzola production where the mold start to growth. This critical phase happened from ripening Day 20, according to the proteolysis data deduced by the *S. thermophilus*-biosensor previously described in the previous chapter. The overall data underlined that the qPCR approach and the *S. thermophilus*-biosensor could represent highly sensitive tools to monitor the industrial productions in a more objective and less operator-dependent way

This method allowed us to unravel some key phases in *P. roqueforti* development, but showed also a low sensitivity for the mould's spores in the first ripening phases. However, this low sensitivity is not related to the primer set, because our observations have confirmed this behaviour also with ITSRoq1 primer set in the first ripening phases. The introduction of the enrichment strategy could be an efficient and not much time wasting method to increase the method sensitivity and its reproducibility, which is now in an improvement phase.

6. Evolution of microbial population dynamics in sweet Gorgonzola cheese and identification of the critical control points that can affect the final cheese quality

6.1. Introduction

The comparison between 2015 and 2016 productions from the same production plant was performed in order to identify the critical control points that could affect the cheese quality. Microbial population dynamics and metabolites evolution during cheese production was performed using the previously described tools in addition to the following analysis.

i) Volatile Organic Compounds (VOCs), which are very important for the organoleptic properties of the cheese, especially for taste and odour characteristics, was measured by the help of SPME GC-MS analysis (Panseri *et al.*, 2014).

ii) Lactose and galactose, the two main sugars available in Gorgonzola cheese production, have been enzymatically quantified, as described in Materials and Methods, to understand their role on *S. cerevisiae* development and fermentative activity, which is relevant for the correct *Penicillium roqueforti* development (Florez and Mayo, 2006).

6.2. Results and Discussion

6.2.1. *P. roqueforti* biomass and cheese proteolysis quantification

P. roqueforti was quantified in 2015 and 2016 productions and the results showed a significant difference between the two productions, being the 2015 the most abundant in terms of fungal biomass (**Figure 15**). In 2016 production, the fungal growth in the critical phase between Day 20 and Day 37 was significantly different from the same samples in 2015 productions. Proteolysis derived from fungal growth is important for the final quality of the product, in particular for the soft texture that characterizes the sweet variety of this cheese, but, on the other hand, an excessive rate of proteolysis could affect the final product shelf-life, because free amino acids at high concentrations

could serve as substrates for biogenic amine formation (Calzada *et al.*, 2013). It has been recently demonstrated in literature that proteolysis from fungal growth gives some important healthy characteristics to this product, like the production of some Angiotensin Converting Enzyme-Inhibitors (ACE-Inhibitors), acting as a vasodilator and an anxiolytic (Stuknyte *et al.*, 2015). **Figure 16** depicts the quantifications of glutamic acid based on the recovery of metabolic activity by *Streptococcus thermophilus* MIM945 biosensor after starvation induced by Aspartic acid, which was related to the glutamic acid or glutamic acid-containing peptides presence: the substrates selected to verify the recovery of metabolic activity were different supernatants obtained by the centrifugation of selected homogenized Gorgonzola samples from both productions, as described in Materials and Methods. Noteworthy, the data obtained using the *S. thermophilus* biosensor and those obtained by the qPCR assay showed a similar trend, except for the differences in terms of fungal biomass and proteolysis detected between Day 20 and Day 60. In this time interval, we measured higher *Penicillium roqueforti* biomass and proteolysis in 2015 samples compared to that from 2016. The lower *Penicillium* growth detected between day 20 and day 37 reflected in a minor quantity with a difference at Day 60 of 74% and at Day 37 of 151% in luminescence production, was detected. The results of these two independent experiments showed a good linear correlation, demonstrating that the qPCR results are in accordance with the luminometric profile obtained (**Figure 17**).

The delayed proteolysis of 2016 samples, probably due to a low *P. roqueforti* growth, was confirmed once again also by the analysis on nitrogen fractions obtained with different types of acid precipitations and depicted in **Figure 18**: in particular, it is noteworthy how, at Day 37, this tendency to delay is concentrated mainly on low molecular weight peptides, that are linked to different flavor descriptors like bitterness and sweetness (Zhao *et al.*, 2016), and that therefore could affect the final product organoleptic properties. Since nowadays the evaluation of Gorgonzola quality during ripening is still made

visually by experienced operators in production plants, there is the need to use this experience to establish thresholds in mycelium development to understand the optimal final quantity of fungal biomass per gram necessary to have a satisfactory product, and use this thresholds to standardize the methods developed in this work.

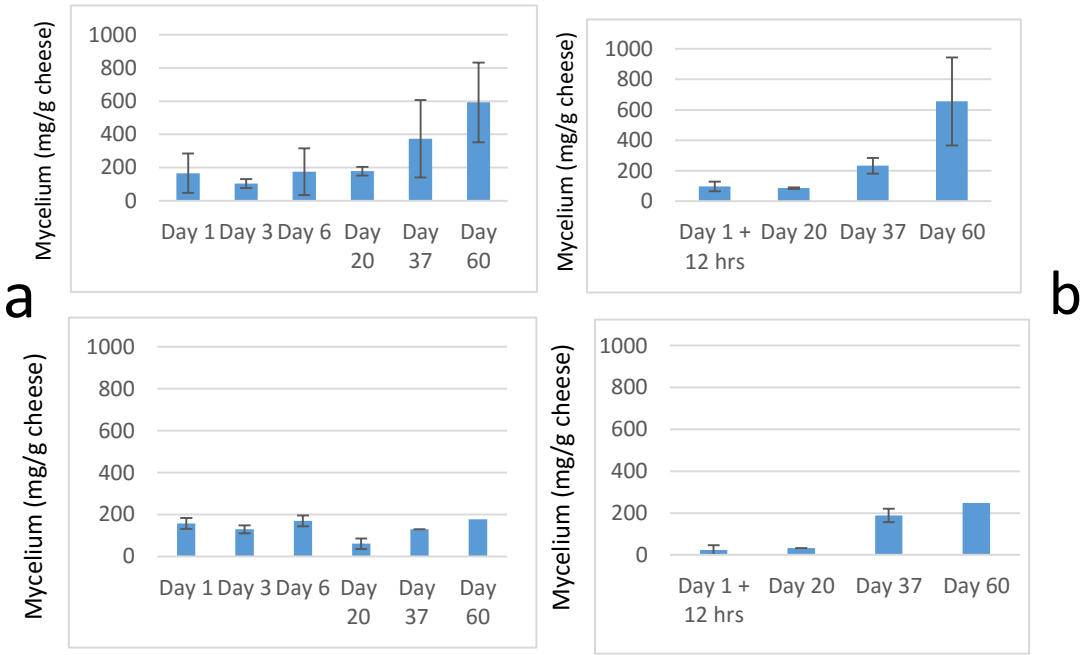

Figure 15: **a**) *P. roqueforti* quantifications performed on paste samples for 2015 (top) and 2016 (bottom) production; **b)** *P. roqueforti* quantifications performed on rind samples for 2015 (top) and 2016 (bottom) production. Each point results from the average between two wheels of the same production ±SD. $0.05 < p < 0.1$.

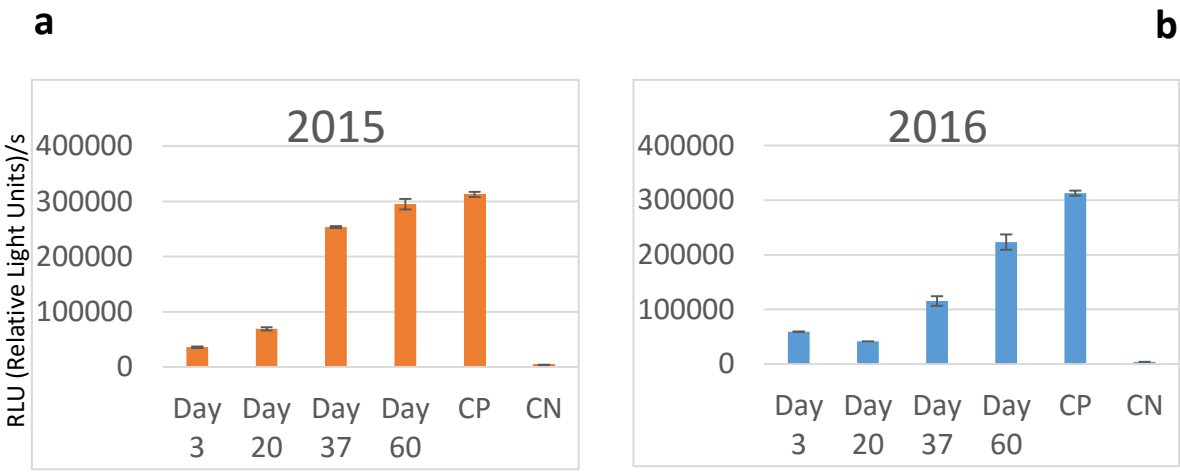

Figure 16: *S. thermophilus*-based biosensor quantifications of glutamic acid-related recovery of metabolic activity by *S. themophilus* pCSS 945 on **a)** 2015 and **b)** 2016 cheese samples. The analysis was performed only on paste samples. Data represent the maximum of light units reached by the samples after 240 minutes of analysis. **CP**, *S.thermophilus* MIM945 supplemented with 2.0 mM Glu; **CN**, *S. thermophilus* MIM945 under aspartic acid induced starvation.

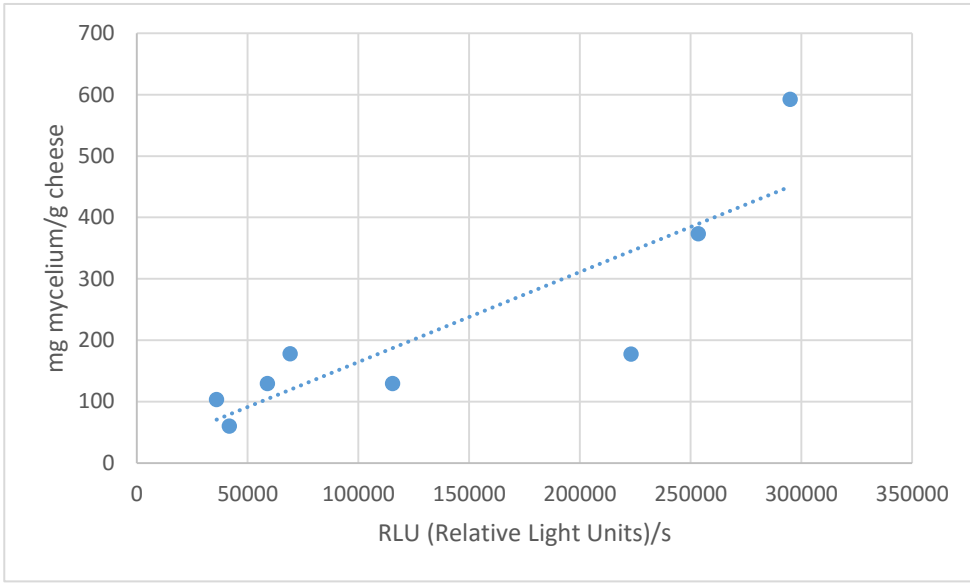

Figure 17: Linear correlation between biomass quantification with qPCR (Y axis) and luminescence produced by metabolic activity recovery (X axis). Pearson Index: 0.7402.

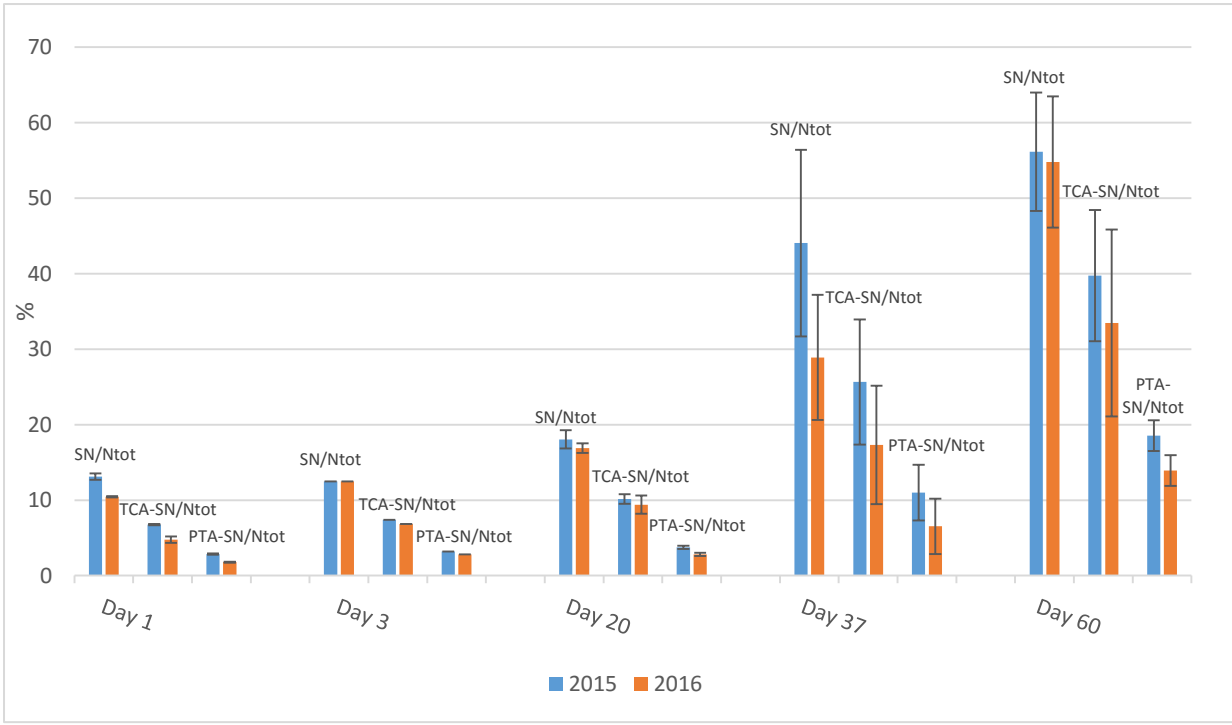

Figure 18: Graphic representation of nitrogen fractions obtained on 2015 **(a)** and 2016 **(b)** cheese samples. **SN/Ntot**: pH 4.4 precipitation for all soluble nitrogen fractions; **TCA-SN/Ntot**: 3kDa peptides ratio with respect to total nitrogen fractions (Trichloroacetic Acid precipitation); **PTA-SN/Ntot**: 600 Da peptides ratio with respect to total nitrogen fractions (Phosphotungstic Acid precipitation). $0.05 < p < 0.1$.

6.2.2. Analysis of Volatile Organic Acids Profiles (VOCs)

One of the most remarkable data that highlighted differences between 2015 and 2016 productions was the relative abundance of some important odor related compounds, detected by GC/SPME-Mass Spectrometry. A remarkable change in the VOCs profile was detected between the two productions. In particular, the difference in relative abundance between important odor descriptors were visible between 2015 and 2016 at the end of ripening (**Figure 19**). Hexanoic and octanoic acids, typically associated to putrid and rancid odor and fundamental for the organoleptic properties of Blue cheeses (Moio *et al.*, 1999) showed a higher level of concentration in 2015 production, thus laying the ground for the hypothesis that there the mold growth was more pronounced, because of a better degradation of desirable fatty acids achieved. On the contrary, 2-octanone, which is associated to a floral and sweet odor, but never associated to Gorgonzola cheese flavor, was detected at higher level in 2016 production. The overall data led us confirmed that 2015 products showed the highest cheese quality in comparison with 2016 ones, better answering to the customer requests and to the severe regulations contained in the PDO certification.

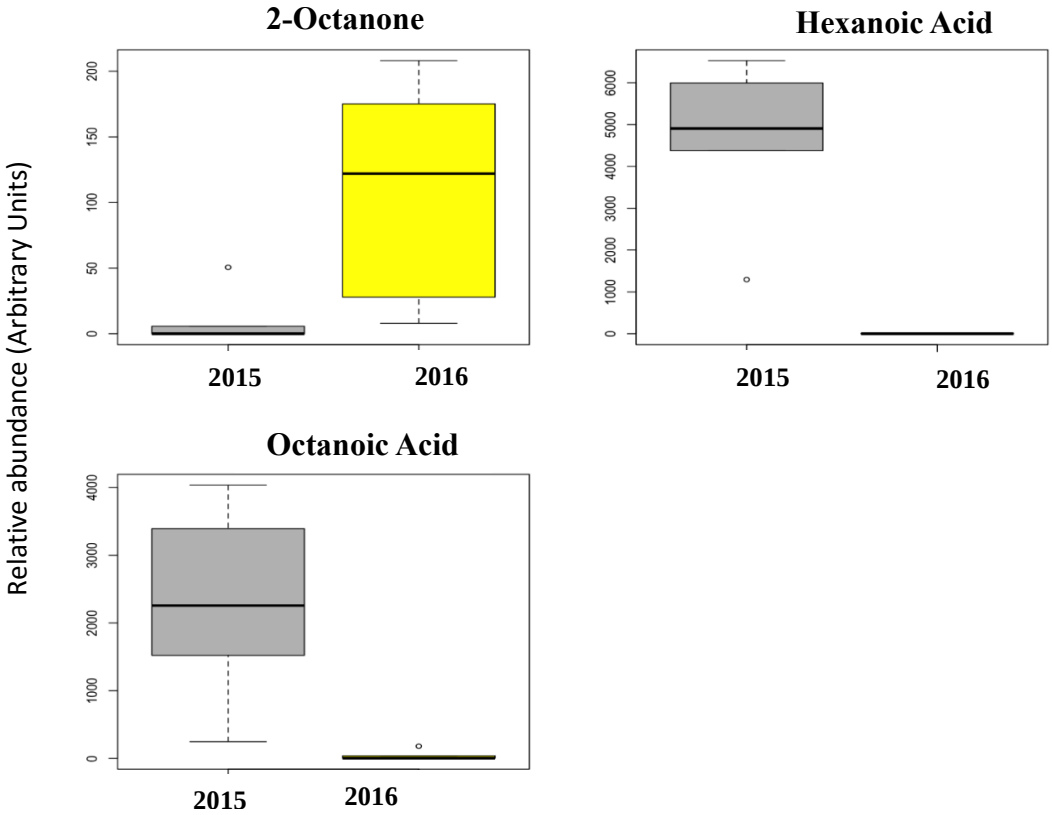

Figure 19: Box Plot representation of the relative abundance (Arbitrary Units) of flavor-related Volatile Organic Compounds in the two productions. Quantitative data obtained with SPME-GC/MS analysis.

6.2.3. Lactose and Galactose Quantifications

Since lactose is the unique carbon source present in the cheese in the first moments of maturation (Ghitti *et al.*, 1995), its initial consumption by Starter Lactic Acid Bacteria is fundamental for the development of the subsequent growing populations. Spectrophotometric measurements on the consumption of this sugar, performed with enzymatic kits, revealed a delay in its degradation by LABs in the first phases of 2016 production (**Figure 20a**). In 2015 samples, lactose concentration dropped from 55,3 to 31,2 g/Kg in the phase comprised between pasteurization and 1 hour after the inoculation, while in the same time frame in 2016 samples, lactose concentration dropped from 47,1 to 43,3 g/Kg. Lactose was not detected in both the production from 9 hours after posing the cheese in Fascera. Measurements performed on galactose consumption as well, confirmed a delay in 2016 samples in comparison with 2015 samples (**Figure 20b**). In particular, when the cheese wheels entered the cabin (22°C), when the best yeast fermentation activity was expected, galactose concentration was significantly higher in 2016 samples in comparison to 2015 (13,6 g/Kg for 2016 production against 8,3 g/Kg for 2015 production), thus suggesting a reduced galactose metabolism in the phases upstream the placing in the cabin.

Despite differences in galactose concentration, where measured, we did not find significant differences in *S. thermophilus* and *L. delbrueckii* counts. However, the higher yeast count measured in Fascera in 2015 production (24 hours before entering in the cabin, **Figure 21**) could justify the differences in galactose content previously discussed. We could therefore speculate that a low galactose degradation rate when the cheese wheel enters the cabin may determine a low carbon dioxide production and a consequent lower holes formation in the cheese paste with a negative effect on the following *P. roqueforti* growth.

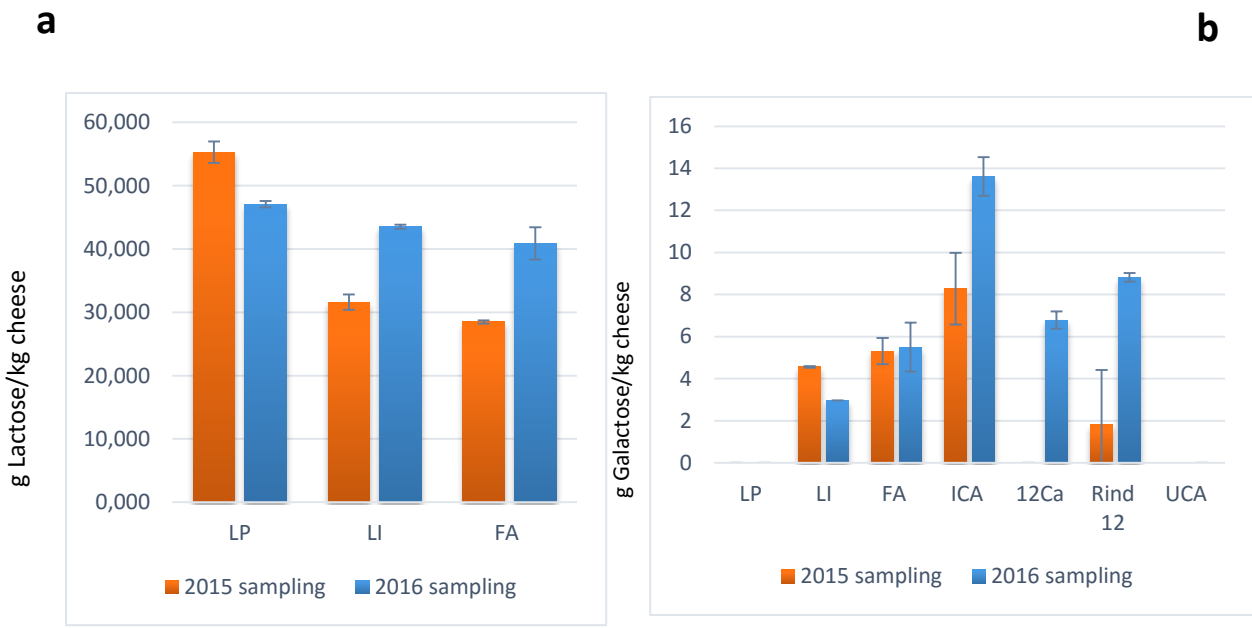

Figure 20: Graphic representation of lactose (**a**) and galactose (**b**) consumption in various ripening phases. Data obtained by the average of the quantifications between two wheels of the same production process ±SD. **Legenda: LP**, pasteurized milk; **LI**, inoculated milk; **FA**, acidified milk; **ICA**, enter in the 22°C cabin; **12Ca**, after 12 hours in the cabin; **Rind 12**, rind after 12 hours in cabin; **UCA**, exit from cabin after 48 hours.

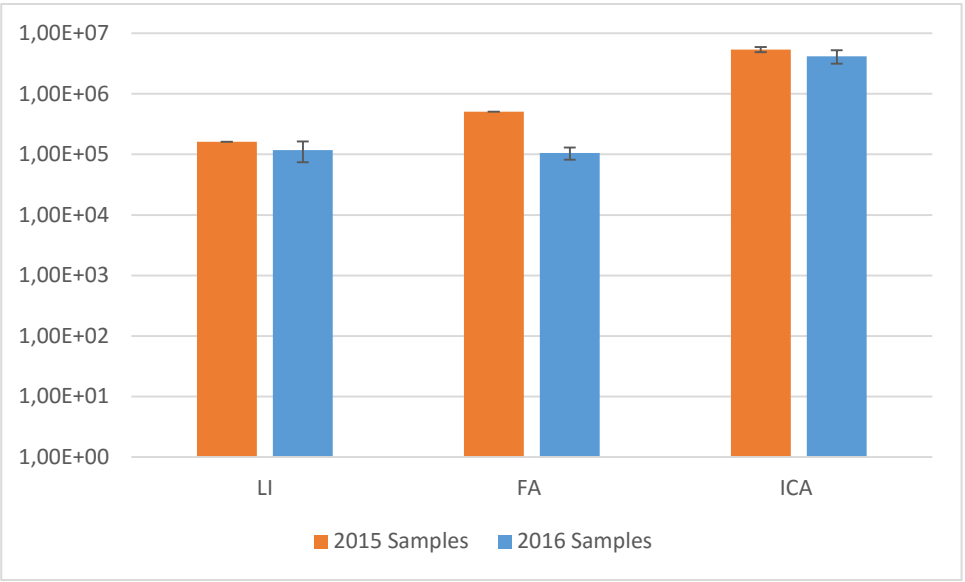

Figure 21: Graphic representation of yeast counts on YGC medium in first production phases, for 2015 and 2016 productions samples. **LI**, Inoculated milk; **FA**, Fascera**; ICA**, Enter in the Cabin. $0.05 < p < 0.1$.

7. Conclusions

In conclusion, the ripening process of sweet Gorgonzola cheese was deeply analysed. The metataxonomic data, coupled with the plate counts during the whole ripening period, have returned a complex picture of the DNA composition of the microbial populations, with substantial differences between productions from different years. The first evidence here was that the process was very influenced not only by the microbial population itself, but also by the environmental conditions of the production plants.

The goal of developing a new method for the following of *Penicillium roqueforti* growth was reached in two coupled ways. The development of a quantitative qPCR assay for the detection of fungal mycelium in samples during ripening provided an efficient and cost-effective method to follow the process in an objective and operator-independent way, with high precision and specificity guaranteed by ARI1 primer set, designed *ad hoc* to amplify only *Penicillium roqueforti* DNA. With this kind of assay, the problems of a long and expensive sample treatment for the ergosterol quantification or of a less precise and timewasting process for the dry mass weight have been replaced with a simple DNA extraction followed by a rapid molecular quantification. With this method, it was possible to monitor all the main stages of product development, and to find a critical step between day 20 and day 37 in which the mould had its strongest blooming, and where it is already possible to address the final product quality by comparing the detectable quantities of fungal mycelium between different productions. Coupling this measurement with the quantification of the proteolysis through the glutamate-sensitive *S. thermophilus* biosensor provided a further integration to the method, confirming that the proteolytic activity of the mould correlate to the biomass quantity reached in a certain ripening moment. The correlation between these two methods was endorsed by analysis on protein fractions and on Volatile Organic Compounds (VOCs) by mass spectrometry that confirmed the previously observed trends.

While the use of the previously described methods unravelled a key phase in mould development in the latter phases of ripening, the metabolic analysis performed with enzymatic kits on lactose and its degradation products – especially galactose – turned out to be useful to understand what kind of first-phase linked parameter could influence the differences between one production and another in terms of biomass amount. Galactose proved to be a very interesting marker to evaluate the quality of the final product, since it was showed that a better development of fungal biomass was directly linked to samples that showed a better galactose degradation rate by *Saccharomyces cerevisiae* in the first two days of production. Since galactose is a product of lactose degradation by LAB, this created a complete link between the four starter species used for Gorgonzola production.

From an industrial and agro-economical point of view, the discovering of the previously mentioned key points in Gorgonzola production pulled out at least two very important indications:

i. Since nowadays Gorgonzola production is still a very traditional process, the novelty introduced by biomolecular and biochemical approaches for the quantification of fungal biomass could permit a more objective and scientific determination of the product invisible characteristics, avoiding a human judgement that is obviously driven by experience, but that, as all human activities, could be afflicted by the human error.
ii. The individuation of these key parameters, in particular of galactose degradation involvement in holes formation by *S. cerevisiae* for the consequent development of *P. roqueforti*, could open the door to new kind of technological shrewdness for the improvement of the production. Precisely, an example could be the selection of new yeast strains with high performance in galactose degradation, in order to intervene upstream of Gorgonzola production.

Lastly, all the characteristics unravelled during this work have led to thinking that Gorgonzola production is a very delicate and complex process, that stands on the subtle balance between all the starter cultures and their activities in the diverse phases, but the knowledge of the previously mentioned key-points and the acquisition of the described techniques could make a fast and production-safe intervention possible.

8. Materials and Methods

Chapter 3 – Microbial populations dynamics in sweet Gorgonzola PDO cheese by ITS and *16S rRNA* gene Profiling

1. DNA Extraction

DNA extraction from samples was carried out with a commercially available PowerFood© DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), with some additional steps designed mostly to increase extraction yield of fungal DNA and cleaning performance of DNA. In particular, 1.8 g of homogenized sample underwent centrifugation (13000 x g for 3 min), the supernatant was discarded, and the pellet was resuspended in 450 µl of a lysis buffer – provided by the supplier. Then, a thermal shocking step was added (heating at 85°C for 30 min followed by freezing at -20°C for 30 min, all steps repeated 3 times), followed by an enzymatic treatment with 400 U of Lyticase (Sigma-Aldrich Italia, Milan, Italy), 1 hour at 40°C, and with 1 mg/ml Proteinase K (Sigma Aldrich), 55°C for 1 night.
After the above mentioned steps the classical protocol supplied by the extraction kit was followed.

2. DNA Sequencing and Metataxonomic Analysis

To identify the different species of microorganisms present in the sample, V4 region of *16S rRNA* (for bacteria identification) and ITS (for yeast identification) has been amplified using a broad-range, specific primer pairs and sequenced. The PCR products obtained has been purified in order to remove primer dimers. Emulsion PCR has been carried out using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies)

according to the manufacturer's instructions. Sequencing of the amplicon libraries has been carried out on a 314 chip using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies) according to the supplier's instructions. The .bam files have been processed using QIIME software, with taxonomy assignment by The Greengenes database version 13_5. In order to calculate downstream diversity measures (alpha and beta diversity), *16S rRNA* and ITS Operational Taxonomic Units (OTUs) have been defined at ≥ 97% sequence homology.

Chapter 4 – Development of a glutamate-sensitive biosensor for the evaluation of proteolytic activity during cheese production

1. Collection of Gorgonzola samples

Gorgonzola samples were obtained from Fratelli Oioli cheesemaker (Cavaglietto, Novara, Italy). For each sampling of solid cheese, paste samples were collected using a sterile conic steel shovel, with which, from each wheel, two cores (approximately 50 g) were taken and mixed together. Liquid samples as milk and coagulated milk (approximately 100 mL) were collected directly from the tanks and conserved into sterile plastic sealed glasses.

All the solid samples (coagulated milk as well) underwent homogenization in 3 volumes of MilliQ distilled water (Millipore Life Science, Billerica, MA, USA), with a Biosystem StarBlender 7500 (Biosystem, Milan, Italy) for 4 minutes at 200 strokes/min. Samples were all freeze-stored at -20°C prior to DNA extraction.

2. Consitution of a bioluminescent system for proteolysis evaluation

S. thermophilus protease negative strain DSM 20617 was purchased by DSMZ microbial collection (Braunschweig, Germany), was maintained in M17 broth (Difco Laboratories, Detroit, MI, USA) at 37°C and then transformed with pCSS945 vector according to what have been previously described by Loimaranta *et al.* (1998). The recombinant strain MIM945 obtained was grown in M17 broth with 4 mg/l chloramphenicol for one night, and then cells were centrifuged at 10.000 *g* for 5 minutes and washed with sterile physiological solution. Washed cells were transferred in 10% reconstituted milk (Difco) with 4 mg/l chloramphenicol and growth stress was induced by addition of 2.0 mM Aspartic acid for 2 hours at 37°C. Cells in milk were subsequently freeze-stored until starting the analysis. 0.2 mM luciferin (Sigma-Aldrich) was added to the solution immediately before starting the analysis.

For preliminary trials with Glutamic acid, different concentrations (0.1, 0.5, 1.0 and 2.0 mM) were added at 1:3 diluted skim milk and mixed with equal volumes of cells solution in a luminometer 96-wells plate.

For trials with Gorgonzola samples, homogenized samples from 2015 and 2016 productions were centrifuged at 9500 *g* for 5 minutes, and supernatants were recovered and freeze-stored until the start of the analysis. A volume of supernatant was then added to an equal volume of starved cells in milk. The recovery of metabolic activity was measured with a Victor^3 Luminometer (Perkin-Elmer Inc., Waltham, MA, USA) at 37°C for 3 or 4 hours.

Chapter 5 – Development of a novel qPCR assay for the quantification of *Penicillium roqueforti* biomass in real time during cheese development

1. Fungal Strains and collection of mycelium for standard constitution

Spores suspentions of *Penicillium roqueforti* PR11, PR14, PR15 and PR18, *Penicillium chrysogenum* and *Geotrichum candidum* (Sacco, Cadorago, Italy) were stored at 4°C. 5 µl of each suspension were layed on a nitrocellulose filter in the center of YGC selective medium plates (20 g liter^{-1} Glucose, 10 g liter^{-1} Yeast Extract, 0.1 g liter^{-1} Chloramphenicol) and grown for 5 days at 25°C (Pitt and Hocking, 1997), until achievement of a good mycelium development.
50 mg of each of the four strains of *P. roqueforti* were excised from the plates with a sterile lancet and weighed: then, to mimic the cheese conditions, they were put together in final volume of 1 g of acidified skim milk at pH 4.5 (Difco, Becton Dickinson and Co., MA, USA) as previously described (Le Dréan *et al.*, 2010). For *P. chrysogenum* and *G. candidum* – to be used as negative controls to test ARI1 specificity to *P. roqueforti* – mycelium was excised from the plates and resuspended in sterile water to then undergo DNA extraction.

2. Primers design

A previously known primer set directed against internal transcribed spacer (ITS) region of *P.roqueforti 18S rRna* gene (Le Dréan *et al.*, 2010) was first used. This first sequence was found using GenBank database. Novel primer set for Aristolochene Synthase (*Ari1*) gene was

designed using full protein characterization and gene sequencing available in literature (Proctor and Hohn, 1993). Sequences were first aligned using Mega4 software (Tamura *et al.*, 2007) and the PCR primers were designed from this alignment using the Primer3 software (Rozen and Skaletsky, 2000), in accordance with the criteria required for qRT-PCR primer design. The primers were purchased from Primm Biotech (Milan, Italy). ARI1 specific primers ARI1F 5' - CCGTTCGTGCACCTTGAAA-3' and ARI1R 5'-ACCTCTGAGAACTTGGCAT-3' were designed to amplify a 223 bp region. As mentioned above ITSroq1F 5′- ACCCCGAACTCTGTCTGAAG-3′ and ITSroq1R 5′-ATTTCGCTGCGTTCTTCATC- 3′ primers were designed to amplify a 106 bp region inside the ITS sequence of *P. roqueforti*.

3. qRT – PCR assays

qRT-PCR assays were performed using a Bio-Rad CFX96 thermocycler on 96-well plates (Bio-Rad Laboratories, Hercules, CA, USA). Each well contained a 15 µl reaction mixture that included 7.5 µl of 2X EvaGreen® PCR Master Mix (Bio-Rad), 0.3 µM of each primer set and 5 µl of DNA sample at convenient dilution. For standard mycelium samples in acidified milk, a standard curve was constructed using 10-fold dilutions (10^{-1} to 10^{-5}) of known amounts of *P.roqueforti* DNA, quantified using a BioTek PowerWave XS2 spectrophotometer (BioTek, Winooski, VT, USA). Total DNA has been extracted according to the previously described protocol. For *P. chrysogenum* and *G. candidum* DNA, a similar method was used to magnify the fungal extraction yield. In the first passage of extraction, 500 µl of water-resuspended mycelium underwent centrifugation and then followed the above mentioned protocol.

For the evaluation of primer set best annealing temperature the following thermal protocol was used: 94°C for 3 min; 94°C for 10 s, 50/58°C for 10 s (with thermal gradient on plate), 72°C for 5 s (35 cycles); and 65°C for 1 min. A melting curve analysis with CFX Manager Software (Bio-Rad) was performed to assure that only one PCR fragment came out from the reaction.

Chapter 6 – Evolution of population dynamics during Gorgonzola cheese production

1. qRT – PCR quantifications on cheese samples

For the qRT-PCR assays on cheese samples another thermal pattern was used: 94°C for 3 min; 94°C for 10 s, 50.7°C for 10 s, 72°C for 5 s (39 cycles); and 65°C for 1 min. Direct quantification of mycelium biomass of the target sample was determined by interpolation of the threshold cycle values (C_t) of each sample in the corresponding standard curve, as previously described (Parladé *et al.*, 2008).

2. Assessment of Proteolysis

Proteolysis was evaluated by measuring the nitrogenous fractions by Kjeldahl method according to an ISO Standard (ISO, 2011). The procedure consisted in the separation of the pH 4.4-soluble nitrogen (**pH 4.4-SN**) from a citrate solution of the cheese. This fraction was further extracted with 12% trichloroacetic acid (**TCA**) or phosphotungstic acid (**PTA**) to obtain the 3 kDa TCA-soluble nitrogen (**TCA-SN**) or the 600 Da PTA-soluble nitrogen (**PTASN**), respectively.

3. Volatile Organic Compound Analysis

Volatile organic compounds were extracted from the headspace of cheese by solid-phase microextraction and analyzed by a GC-MS procedure according to Masotti *et al.* (2017), slightly modified for better evaluation of the acidic fraction. Briefly, 1 mL of a 0.12 *M* H2SO4 solution was added to 5 g of cheese along with 2 g of NaCl immediately before volatile extraction, all other parameters being the same previously described. Semiquantitative evaluation of VOC was carried out by integrating the peak area of the characteristic ion (Qion) using the MS-Chemstation software (Agilent Technologies, Santa Clara, CA). Data are the mean values of 2 replicates.

4. Enzymatic kits analysis

Quantifications on lactose and galactose were obtained using enzymatic kits (R-Biopharm Italia, Milan, Italy), according to the producer instructions. Prior to the analysis samples underwent a Carrez clarification procedure (IDF Standard, 1991) to avoid the interference of milk proteins and fat layers with the spectrophotometric analysis; in particular, a starting quantity of 5 g of each homogenized sample was added to 2.5 ml of 85 mM potassium hexacyanoferrate (II) (Carrez-solution I), 2.5 ml of 250 mM zinc sulfate (Carrez-solution II) and water to the final volume of 50 ml. After the addition of the two Carrez-solutions pH was adjusted to 7.5-8.5 with 0.1 M sodium hydroxide. The solutions obtained were finally filtered with a nitrocellulose filter and the flow-through was used undiluted for the quantifications. Spectrophotometric analysis were performed on a Bio-Rad SmartSpec™ Plus instrument (Bio-Rad Italia, Milan, Italy).

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9. Products

BMMIB – Behind Microbe-Microbe Interactions in Blue Cheeses

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State of the art

The Blue cheese are mainly characterised by the use of the mould *Penicilium roqueforti* during the production process, which confers them a typical appearance and flavour. Gorgonzola is the most famous Italian blue cheese, its tradition is very long in Italy and the first evidences of Gorgonzola production are dated back to 879 b.c. in Gorgonzola, nearby Milan. It is made of pasteurized cow's milk inoculated with thermophilic Lactic Acid Bacteria (LAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Ariolli *et al.*, 2007). LAB work as starter cultures during the beginning of ripening. It has been shown that the initial growth and production of lactic acid by thermophilic bacteria is necessary for the development of the environmental conditions (low pH and lactic acid as a carbon source) suitable for the rapid growth of yeast (commonly *Saccharomyces cerevisiae*) and *Penicilium roqueforti* (Flórez and Mayo 2006; Cheeseman *et al.*, 2014), which is responsible for the main features of this cheese. As the popularity of blue cheeses is on the rise, more interest have been focused on the scientific characterisation of the cheese microbiota. Indeed, the first objective of an economical plan investing in this field is studying/measuring the parameters/values influenced by microorganisms to find out the optimal conditions in order to obtain a safe and qualitatively better product. For example, the ability of pathogens to grow in cheese is largely dependent on the manufacturing steps required for cheese-making (extent of acidification by the starter culture, the correct temperature to be applied at various stages during the manufacture, ripening/maturation conditions), the physicochemical characteristics of the cheese (pH, salt content, water activity), and the growth requirements of the microorganism.

Aim of the project

The aim of this Ph.D. project is to clarify the principal role of each microorganism in each phase of the cheese production in order to offer a detailed description of the community composition and to describe the role of each species in production of an high quality Gorgonzola cheese. For this purpose metagenomics and metatranscriptomic approaches will be coupled with well-known culture-dependent and chemical methods to characterize the blue cheese microbiota at each stage of production. Moreover, since microbiological interactions are pivotal for the features of the final product, LAB-yeasts-*Penicilium* interactions will be studied *in vitro* and *in vivo*. Then, a SYBR green-based qRT-PCR assay and flow cytometry assay will be set out as quantitative methods to monitor each component of the cheese microbiota, including the mycelium (hyphae) of the fungal population during cheese production.

PhD Thesis Objectives and Milestones:

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities, according to the Gantt diagram given in Table 1 and sketched in Figure 1:

A1. Microbiota characterization by culture-dependent methods: the aim is to find out which are the best cultural conditions and parameters that are suitable for the good growth of LAB-Yeasts-Moulds.

A2. First approach with the Gorgonzola samples: sampling and chemical/microbiological analysis on milk and Gorgonzola matrixes at each stage of production; set up of DNA extraction and design and evaluation of primer sets species-specific for *Penicillium roqueforti* in order to develop a quantitative-PCR assay useful to follow the fungal biomass growth during cheese production.

A3. Development of metagenomics and metatranscriptomic approaches: in order to follow the population growth in paste and rind, and developing a lab-scale Blue-Cheese model of the Gorgonzola production.

A4. Development of a lab-scale Blue-Cheese model: this step should be very important to study the possible presence and the biocontrol strategies against food pathogens associated to blue-cheeses (*e.g. Listeria monocytogenes*).

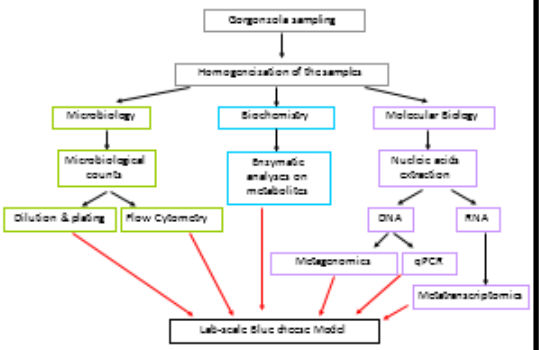

Figure 1: schematic representation of each step of my Ph.D. project

Activity \ Month	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	35	36
Microbiome characterization and gorgonzola sampling (A1+A2)	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■																		
Development of metagenomic and metatranscriptomic approaches (A3)												■	■	■	■	■	■	■	■	■	■	■	■	■												
Development of a lab-scale Blue cheese model (A4)																		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Preparation of manuscripts and thesis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

Table 1: Gantt diagram for this Ph.D. project

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XX WORKSHOP
ON THE DEVELOPMENTS IN THE ITALIAN PHD RESEARCH ON FOOD SCIENCE,
TECHNOLOGY AND BIOTECHNOLOGY

PERUGIA, SEPTEMBER 23rd-25th 2015

Monitoring of *Penicillium roqueforti* development during Gorgonzola cheese ripening

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State of the art. Gorgonzola is a blue-veined, mould-ripened cheese, made from pasteurized cow's milk inoculated with starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii*) along with *Saccharomyces cerevisiae* and *Penicillium roqueforti*, the main responsible for the aroma and flavor of the cheese at the end of ripening. For its importance in the final product quality, monitoring the fungal growth during the ripening process is crucial. Traditional methods based on colony forming unit (CFU) are not suitable for hyphal filaments quantification in the cheese matrix, and neither alternative ones, like indirect ergosterol quantification [Dong *et al.*, 2006]. For all these reasons there is the need for a novel type of approach for the quantification of fungal biomass in real-time during ripening and in this project we found it in a brand new qRT-PCR approach, using a novel primer set directed against Aristolochene synthase (*Ari1*) gene of *P. roqueforti* [Proctor & Hohn, 1993]. These molecular analysis were coupled with biochemical data from mass-spectrometry to try to correlate the chemical changes in the matrix with the fungal growth and build a real combined qualitative-quantitative method for the detection of *P. roqueforti* biomass development during all ripening phases.

qPCR trials with Ari1 primer set. qPCR quantification of *P. roqueforti* mycelium using the novel primer set Ari1: the melting curve profiles disclosed the presence of a transition phase from Day 20 to Day 27, before which the melting signals are very soiled and overlapped [Figure 1a]. The hypothesis was that the natural signal cleaning achieved after Day 27 [Figure 1b] was due to a stronger increase of *P. roqueforti* growth.

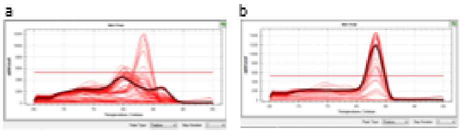

Figure 1: melting curves from qPCR quantification on Gorgonzola samples with Ari1 primer set; a) soiled samples from start of ripening to Day 20: the well-defined melting peak that are visible are the highlighting in standard curve samples; b) clean samples from Day 27 to the end of ripening.

Signal "cleaning" and final mycelium quantification. Signal Cleaning trials in order to correctly quantify fungal mycelium also in the first ripening phase. 5 ng of exogenous pure *P. roqueforti* DNA were used to enrich soiled samples, and achieved a complete cleaning of the specific Ari1 melting curve [Figure 2a]. This allowed the correct quantification of the fungal mycelium during the whole ripening time [Figure 2b].

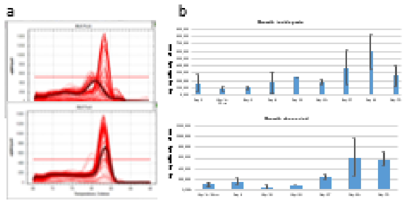

Figure 2: a) comparison between melting profiles for a young Gorgonzola sample without (top) and with (bottom) 5 ng of pure exogenous *P. roqueforti* DNA added in reaction; b) quantification on a few cheese samples and dried samples.

UPLC-MS analysis on peptidic profile. The Mass Spectrometric analysis revealed a visible simplification of the peptidic profile from the first phase until the second one associated to a strong *P. roqueforti* growth [Figure 3], with particular attention to the disappearance of peptides typically associated to bitter taste; the only bitter components that are stable during the whole ripening are aminoacids like phenylalanine and tyrosine – highlighted with blue arrows.

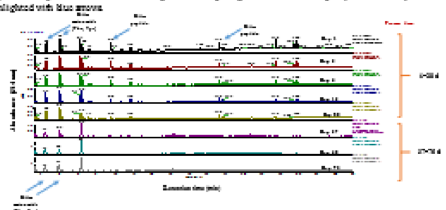

Figure 3: Mass Spectrometry plot on the peptidic profile in the analysed ripening phases.

Identification of ACE-Inhibitor peptides. In the transition between the two phases a remarkable increase in the quantity of Angiotensin Converting Enzyme-Inhibitor peptides, due to the intense proteolytic action of *P. roqueforti*, was observed [Table 1]. These peptides have a great biological value as they have ancyolytic properties.

Sampling time point	VPP	IPP	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Total
(d)					(mg kg⁻¹)				
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	nd	[illegible]	nd	[illegible]	[illegible]
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[illegible]	[illegible]	[illegible]	[illegible]	nd	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	nd	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Cheese ripening age is expressed in days and indicated in brackets.
Values are presented as mean of two samples ± SD.
nd, not detected.

Table 1: Levels of ACE-I peptides in Gorgonzola cheese fractionated (<3 kDa) water-soluble extracts (WSEs).

Volatile Organic Acids (VOCs) Profile. A remarkable change in the VOCs profile was detected in the same period. In particular, the transition between the lactic acid bacteria-related phase to the *P. roqueforti*-related phase is linked to the relative abundance of some important taste and odour descriptors increase [Fig 4], with particular attention to heptanone and nonanone, which are associated to typical "*cheesy*" flavour, and to octanoic and butanoic acid which are typically associated to rancid and sweaty odour and fundamental for the organoleptic properties of Blue cheeses [Moio *et al.*, 2000].

Figure 4: Box Plot representation of the relative abundance (Arbitrary Units) of various flavour-related Volatile Organic Compounds in the two key phases of ripening. Quantitative data obtained with Aldrich/TD-GC-MS analysis.

Conclusions. The analysis performed with qPCR molecular approach showed a great increase of *Penicillium roqueforti* mycelium growth from Day 27 on, with a major clearness in the detection of the specific *P. roqueforti* signal from this moment until to the end of ripening: in this phase the quantification didn't need any signal "cleaning" attempt. The presence of a key phase in the maturation between Day 20 and Day 27 was confirmed also by UPLC-MS and GC-MS data, respectively for the proteolysis and for the Volatile Organic Compounds profiles. The simplification of peptidic profile and the total change of VOCs profile in this transition period confirmed what we obtained with qPCR data: a great increase in *P. roqueforti* mycelium growth, that reflects in a massive increase in the enzymatic activity of this mould.

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BMMIB-Behind Microbe-Microbe Interactions in Blue cheeses

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State of the art Gorgonzola is a blue-veined, mould-ripened cheese, made from pasteurized cow's milk inoculated with starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii*), along with *Saccharomyces cerevisiae* and *Penicillium roqueforti*, the main responsible for the aroma and flavor of the cheese at the end of ripening. The microbial interactions that occur during the ripening process are crucial for the correct development of the final product, which is severely controlled to fulfill the standards of its PDO denomination. These interactions have been studied poorly [1,2] in the past years: for this reason the aims of this work are i) the characterization of the evolution of microbial populations in each phase of ripening, using traditional microbiological approaches, together with a metagenetics analysis, ii) the enzymatic quantification of microbial metabolites pathways involved during cheese ripening, together with mass-spectrometry analysis and iii) the development of a species-specific primer set for the quantification of *Penicillium roqueforti* mycelium [3] by qPCR during cheese ripening.

Microbial ecology of cheese samples. The evolution of the microbiota components during the cheese production process was evaluated by viable counts on three different selective media: M17 for Streptococci, MRS (pH 5.4) for Lactobacilli, and YGC for Yeasts [Fig. 1]. Samples belonging to most crucial ripening phases were then subjected to a metagenetics analysis based on the *16SrRNA* gene (Fig. 2a, for prokaryotes), and on the ITS portion of *18SrRNA* (Fig. 2b, for eukaryotes).

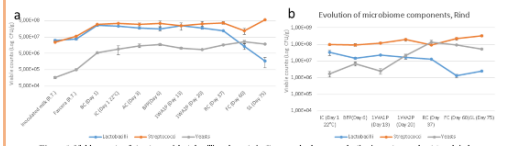

Figure 1: Viable counts of streptococci, lactobacilli and yeasts in Gorgonzola cheese production:in paste samples (**a**) and rind samples (**b**). Data are the average of two samples collected from different cheese wheels ± SD.

Figure 2: Metagenetics, represented at species level, as calculated from *16SrRNA* gene (**a**) and ITS (**b**) profiling. Only taxa with relative abundance > 1% are represented.

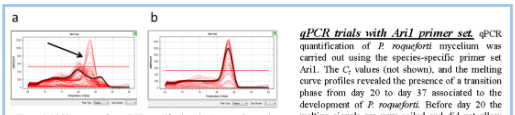

Figure 3: Melting curves from qPCR quantifications in Gorgonzola samples using the Ari1 primer set; **a**) samples collected from day 1 to day 20; **b**) from day 37 to the end of ripening. The arrow indicates the melting curves obtained using pure *P. roqueforti* DNA as template.

qPCR trials with Ari1 primer set. qPCR quantification of *P. roqueforti* mycelium was carried out using the species-specific primer set Ari1. The C_t values (not shown), and the melting curve profiles revealed the presence of a transition phase from day 20 to day 37 associated to the development of *P. roqueforti*. Before day 20 the melting signals are very soiled and did not allow the *P. roqueforti* quantification [Fig. 3a]. We therefore conclude that *P. roqueforti* DNA extracted from cheese paste and rind was detectable (>5 ng) from day 37 [Fig. 3b].

Increasing qPCR sensitivity and final mycelium quantification. 5 ng of exogenous pure *P. roqueforti* DNA were used to enrich soiled samples in order to quantify low amount of *P. roqueforti* DNA [Fig. 4a] before Day 37. This allowed the quantification of the fungal mycelium during the whole ripening time [Fig. 4b].

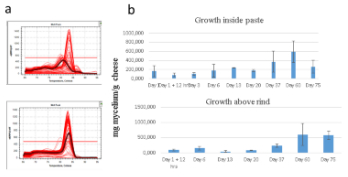

Figure 4: **a**) Comparison between melting curves from qPCR quantification of Gorgonzola samples without (top) and with (bottom) 5 ng of exogenous pure *P. roqueforti* DNA added in reaction; **b**) qPCR quantifications of mycelium in paste and rind samples.

Quantification of microbial metabolites. Microbial metabolites were quantified enzymatically (R-Biopharm, Italy). Ethanol production was detectable in the cheese paste 24 h after the wheels preparation and prior to the switch the temperature from 4 °C to 22 °C. Interestingly, Ethanol amount decreased both paste and rind after 20 days of ripening. Lactic acid decreased after 37 days of ripening, and mainly in cheese rind. Both the decrease in Ethanol and lactici acid could be related to the development of *P. roqueforti*, and the formation of a complex microbial community in the cheese rind.

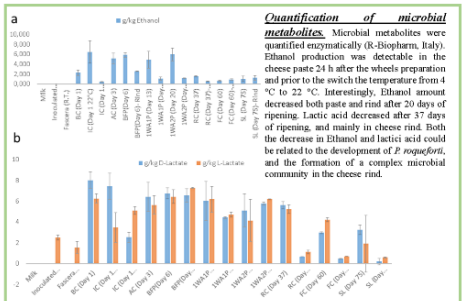

Figure 5: Quantification of ethanol (**a**) and D,L-Lactic acid (**b**) during cheese production. Data are the average of two samples collected from different cheese wheels ± SD.

UPLC-MS analysis on peptidic profile. The Mass Spectrometric analysis revealed a visible change and simplification of the peptidic profile from ripening day 20 to day 37 [Fig. 6], with particular attention to the disappearance of peptides typically associated to bitter taste; the only bitter components that are stable during the whole cheese ripening are the aminoacids phenylalanine and tyrosine whose signals are highlighted with blue arrows in the figure.

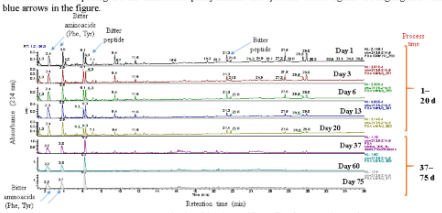

Figure 6: Mass Spectrometry plot describing the peptidic profile of Gorgonzola samples.

Volatile Organic Acids (VOCs) Profile. A remarkable change in the VOCs profile was detected in the same period. In particular, the transition between the lactic acid bacteria-related phase (from day 1 to day 20) to the *P. roqueforti*-related phase (from day 37 to day 75) is linked to the increase in the relative abundance of some important taste and odour descriptors [Fig.7]. It is worth of mention the change in relative abundance of **hexanone** and **heptanone**, which are associated to typical ***"cheesy"*** flavour, and of **octanoic** and **butanoic** acid, which are typically associated to ***putrid*** and ***rancid*** odour, and fundamental for the organoleptic properties of several Blue cheeses [4].

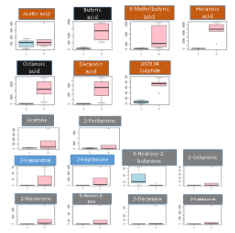

Figure 7: Box Plot representing the relative abundance (Arbitrary Units) of flavour-related Volatile Organic Compounds in the two key-phases of ripening. Quantitative data obtained with SPME-MS analysis on paste samples. **1** = non-mature samples (from day 1 to day 20); **7** = mature samples (from day 37 to day 75).

Conclusions. In this first deep study on the microbiological evolution and interactions inside Gorgonzola cheese during ripening, many remarkable data have been produced. The plate count data mostly provided expected results, and they showed a large increase of yeast numbers before the incubation of cheese wheels into the cabin, where the yeast activity is expected to produce the highest level of carbon dioxide necessary for the formation of the «caverne»; After the preparation of cheese wheels, the internal temperature of each wheels remains relatively high (28-30 °C) for several hours, thus allowing the growth of the yeast that was energetically based on the galactose released by *S. thermophilus* and *L. delbrueckii* lactose fermentation. The metagenetics data based on the *16SrRNA* gene profiling revealed the presence of a large abundance of *Cellulosimicrobium cellulans* DNA, especially on the rinds according to previous observations [1]; otherwise we showed how the presence of high relative amount of *C. cellulans* DNA was found in the pasteurized milk too, thus indicating the probable contamination of the pasteurizing plant with this microorganism. In this context the presence of *C. cellulans* DNA in milk will be further investigated. The metagenetics ITS data revealed the greater abundance of *Debaryomyces hansenii* among the yeast population in the rind samples rather than in the paste, which is a common feature for blue cheeses [2]. A key phase in cheese ripening was observed between day 20 and day 37, as deduced by peptides and volatile organic compounds (VOCs) profiles, and qPCR quantification of *P. roqueforti* mycelium. The simplification of peptidic profile, and the total change of VOCs profile in this transition period was associated to the start of spore germination and growth of *P. roqueforti*, and the relative increase in its proteolytic activity.

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Penicillium roqueforti development during Sweet Gorgonzola cheese production

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State of the art Gorgonzola is a blue-veined, mould-ripened cheese, made from pasteurised cow's milk inoculated with starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii*), along with *Saccharomyces cerevisiae* and *Penicillium roqueforti*, the main responsible for the aroma and flavor of the cheese at the end of ripening. For its importance in the final product quality, monitoring the fungal growth during the ripening process is crucial. Traditional methods based on colony forming unit (CFU) are not suitable for hyphal filamentous quantification in the cheese matrix, and neither alternative ones, like indirect ergosterol quantification (Dong *et al.*, 2006). For all these reasons there is the need for a novel type of approach for the quantification of fungal biomass in real-time during ripening and in this project we found it in a brand new qPCR approach, using a novel primer set directed against arsenicoheme synthase (*Ars1*) gene of *P. roqueforti* (Proctor & Hohn, 1992). These molecular analysis were coupled with an assay with a glucose-sensitive luminescent biosensor for the evaluation of proteolysis along with biochemical data from mass-spectrometry and enzymatic kits analysis on sugar consumption, to try to correlate the chemical changes in the matrix with the fungal growth during two productions from two different years and build a real combined qualitative-quantitative method for the detection of *P. roqueforti* biomass development during all ripening phases.

qPCR quantifications of P. roqueforti

qPCR quantification of *P. roqueforti* mycelium using the novel primer set *Ars1*: the growth profiles, along with the melting curves data (data not shown), disclosed the presence of a transition phase from Day 20 to Day 27, after which the mould has a strong blooming. This blooming appeared to be more significant in 2015 production in comparison with 2016 production, both in paste (Figure 1a) and rind samples (Figure 1b)

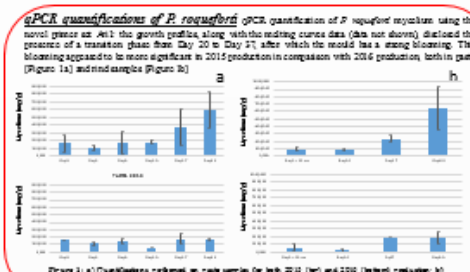

Figure 1: a) Quantifications performed on paste samples for both 2015 (top) and 2016 (bottom) productions; b) quantifications performed on rind samples for both 2015 (top) and 2016 (bottom) productions. Each point results from the average of quantifications between two heads of the same production process ± SD.

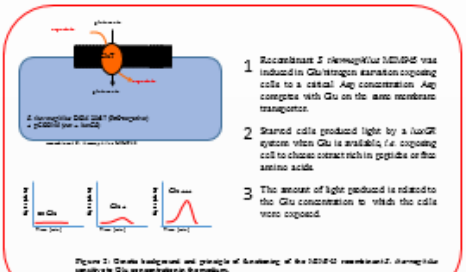

Figure 2: Genetic background and principle of functioning of the NISIN-GI recombinant *L. thermophilus* sensitive to Glu concentrations in the medium.

Evaluation of cheese proteolysis using a S. thermophilus biosensor

Exposing a cheese extract to the recombinant *S. thermophilus* pCSS 945-Glu-sensitive, it was possible to monitor the proteolytic activity associated to the growth of *Penicillium roqueforti*. The results obtained showed higher level of proteolysis in 2015 cheese production, compared to the 2016 production, from day 20 to day 40 (Figure 3a). These results were in agreement with *P. roqueforti* qPCR quantification showed in Figure 1.

Figure 3: Luminescence quantification on glucose-related recovery of metabolic activity by *S. thermophilus* CSS 945 on a) 2015 and b) 2016 cheese samples. Analysis performed only on paste samples. Data represent the maximum of light units reached by the samples after 240 minutes of analysis. CF, *S. thermophilus* pCSS 945 with 1 mM Glu; CN, *S. thermophilus* pCSS 945 without Glu.

Analysis of cheese nitrogen fractions

The evaluation of nitrogen fractions obtained with different types of precipitations confirmed once again the delayed proteolysis of 2016 samples (Figure 4B) in comparison with 2015 samples (Figure 4a), between Day 20 and Day 27; in particular, it is noteworthy how this delay is concentrated mainly on low molecular weight peptides, that are linked to different flavor descriptors like bitterness and sweetness.

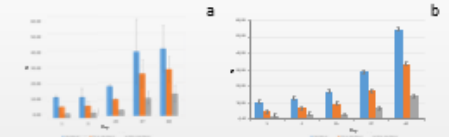

Figure 4: Graphic representation of nitrogen fractions obtained on 2015 (a) and 2016 (b) cheese samples. SN/Tot: pH 4.6 precipitation for all soluble fractions; TCA-SN/Tot: 0.12g/ml precipitation rate with respect to total nitrogen fractions (Trichloroacetic Acid precipitation); PTA-SN/Tot: 0.2g/ml precipitation rate with respect to total nitrogen fractions (Phosphotungstic Acid precipitation).

Volatile Organic Acids (VOCs) Profile

Figure 5: Bar Plot representation of the relative abundance (Arbitrary Units) of the annotated Volatile Organic Compounds in the two productions. Quantification was performed with Xcalibur Qual Browser.

A remarkable change in the VOCs profile was detected between the two productions. In particular, the difference in relative abundance between important odour descriptors was visible between 2015 and 2016 (Figure 5). In specific, 2-octanone, which is associated to a floral odour was detected at higher levels in 2016 production, whereas hexanoic and octanoic acids, typically associated to *rancid* and *vomit* odour and fundamental for the organoleptic properties of Blue cheese (Moio *et al.*, 2000) showed a higher level of concentration in 2015 production where the mould growth was more pronounced.

Lactose and galactose quantification

Sugars quantification with enzymatic kits (R-Biopharm, Germany) has revealed a delay in the consumption of lactose and in the simultaneous production of galactose in the 2016 production compared with the production of 2015 (Figure 6). We hypothesize that the lower amount of galactose available in the 2016 production have determined a lower carbon dioxide production by the galactose-consumer *S. cerevisiae* and a consequent lower holes formation in the cheese paste with a negative effect on the following *P. roqueforti* growth.

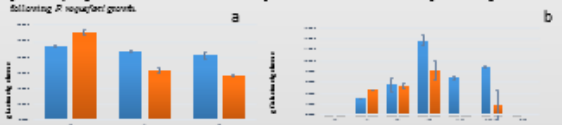

Figure 6: Graphic representation of Lactose and Galactose consumption in various ripening phases. Data obtained by the average ± SD quantifications between two wheels of the same production process at 20 days. Legends: LF, pasteurised milk; LI, inoculated milk; PA, acidified milk; RCA, ripener in the 12°C salter; 12Ca, after 12 hours in the salter; Rind 12, rind after 12 hours in salter; UCA, end ripener after 60 days.

Conclusions

The overall data collected showed that sweet Gorgonzola cheese production is a complex process whose success depend on the metabolic activity of all the microbial actors which must act in perfect timing. The main role of lactic acid bacteria is related to the production of lactic acid and more importantly to the release of galactose which is essential for the growth of *S. cerevisiae* and carbon dioxide production which is fundamental for the holes formation. Holes formed by carbon dioxide are then essentials, once replaced by oxygen after the cheese piercing, to allow the optimal growth of *P. roqueforti*. The qPCR-protocol developed here and the recombinant *S. thermophilus* biosensor were both useful tools respectively for *P. roqueforti* quantification and the conversion of *P. roqueforti* proteolysis during cheese ripening, suggested by the Volatile Organic Compounds profiles obtained by mass spectrometry.

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