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 *16S rRNA* gene (Fig. 2a, for prokaryotes), and on the ITS portion of *18S rRNA* (Fig. 2b, for eukaryotes).

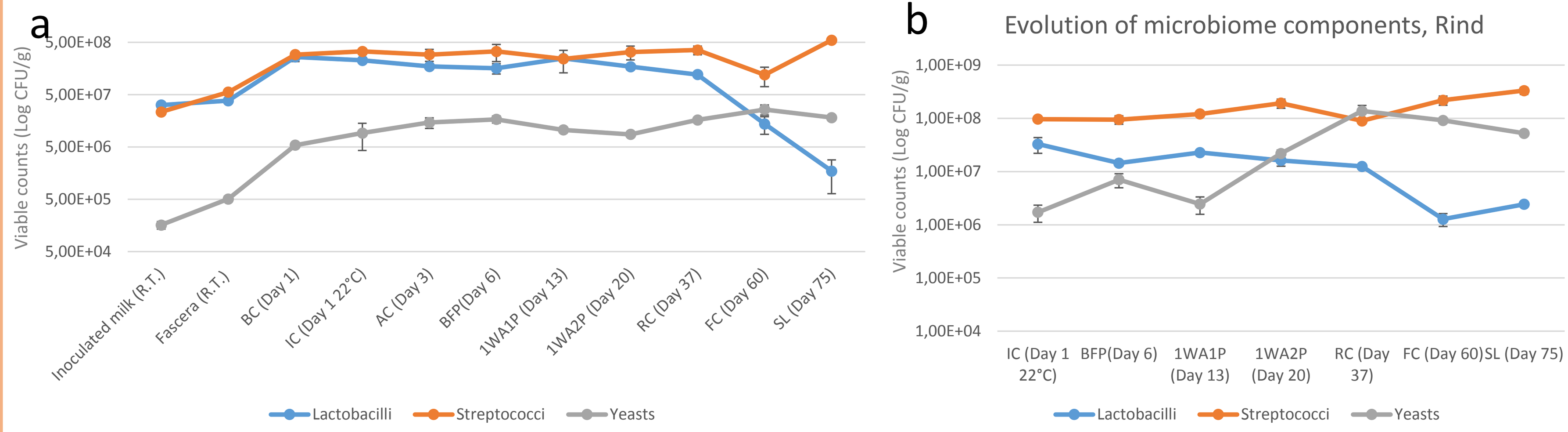

Figure 1: Viable counts of streptococci, lactobacilli and yeasts in Gorgonzola cheese production in paste samples **(a)** and rind samples **(b)**. Data are the average of two samples collected from different cheese wheels ± SD.

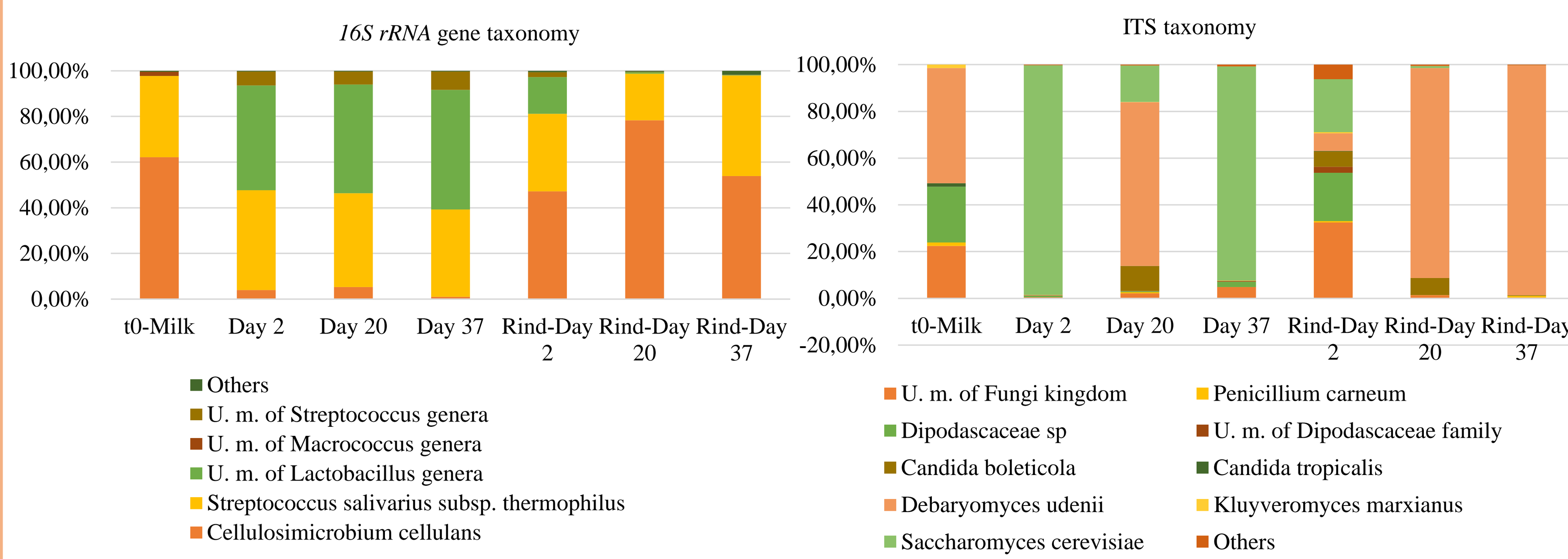

Figure 2: Metagenetics, represented at species level, as calculated from *16S rRNA* gene **(a)** and ITS **(b)** profiling. Only taxa with relative abundance > 1% are represented.

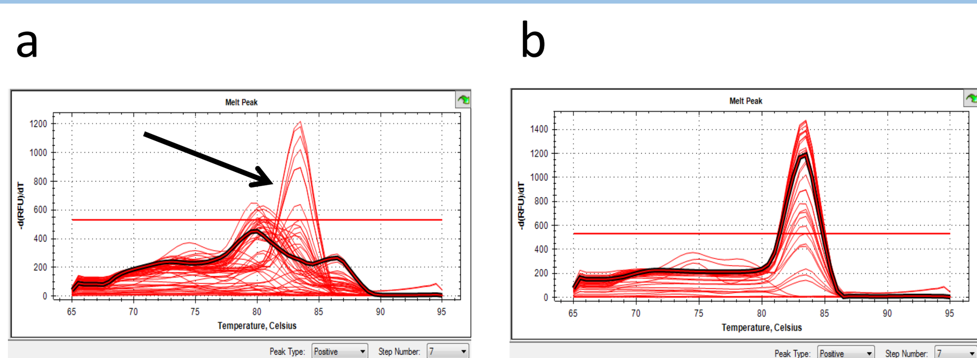

Figure 3: Melting curves from qPCR quantifications in Gorgonzola samples using the Ari1 primer set; **a)** samples collected from day 1 to day 20; **b)** from day 37 to the end of ripening. The arrow indicates the melting curves obtained using pure *P. roqueforti* DNA as template.

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].

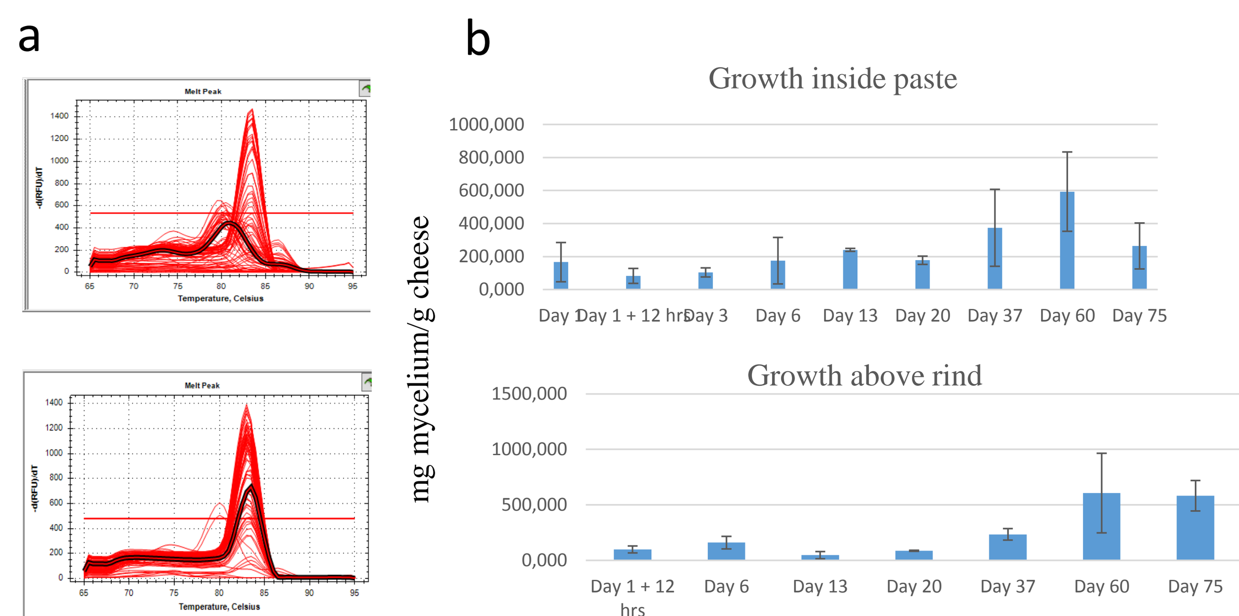

Figure 4: **a)** Comparison between melting curves from qPCR quantification of Gorgonzola samples without (top) and with (bottom) 5 ng of exogenous pure *P. roqueforti* DNA added in reaction; **b)** qPCR quantifications of mycelium in paste and rind samples.

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.

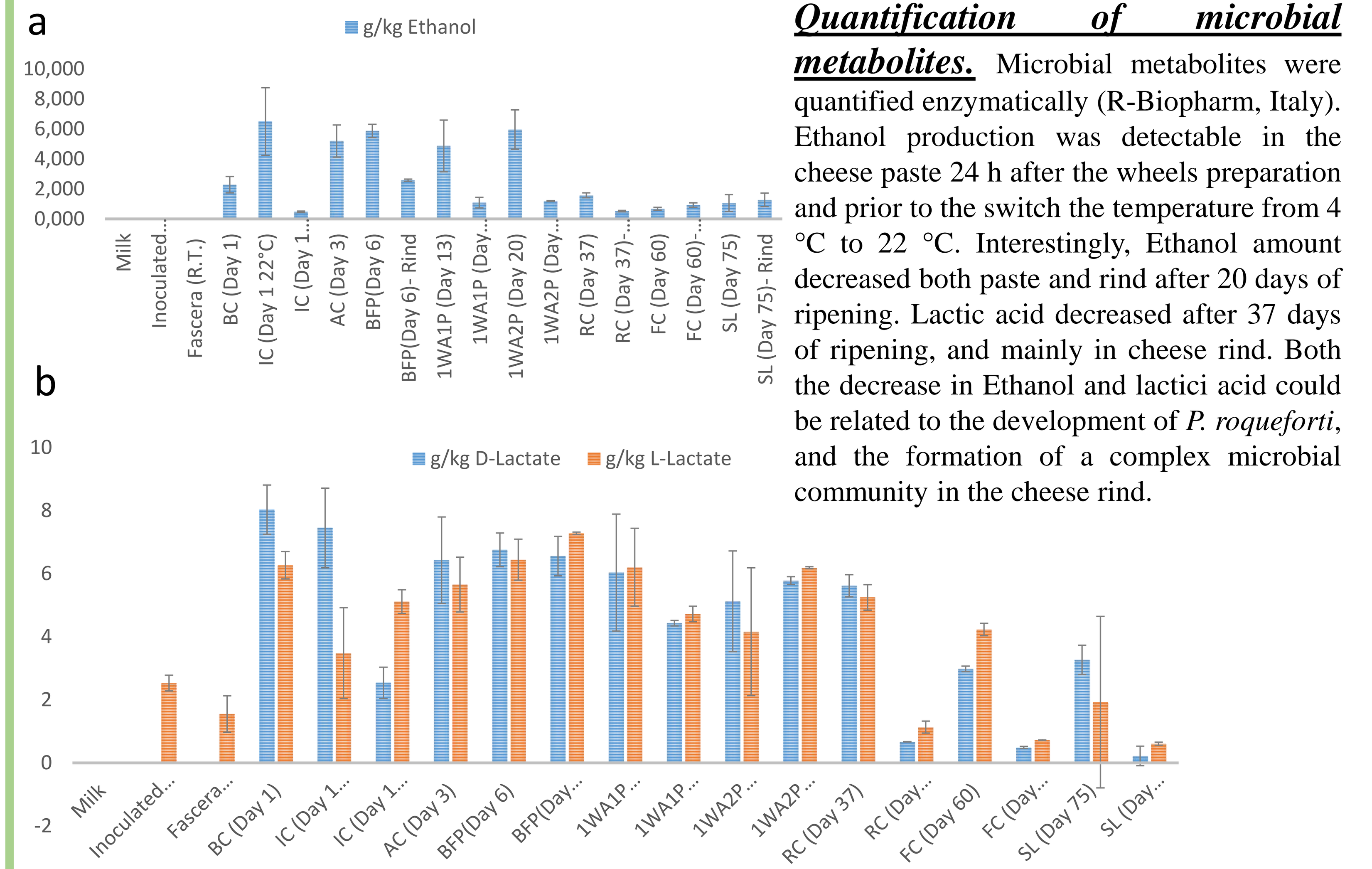

Figure 5: Quantification of ethanol **(a)** and D,L-Lactic acid **(b)** during cheese production. Data are the average of two samples collected from different cheese wheels ± SD.

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.

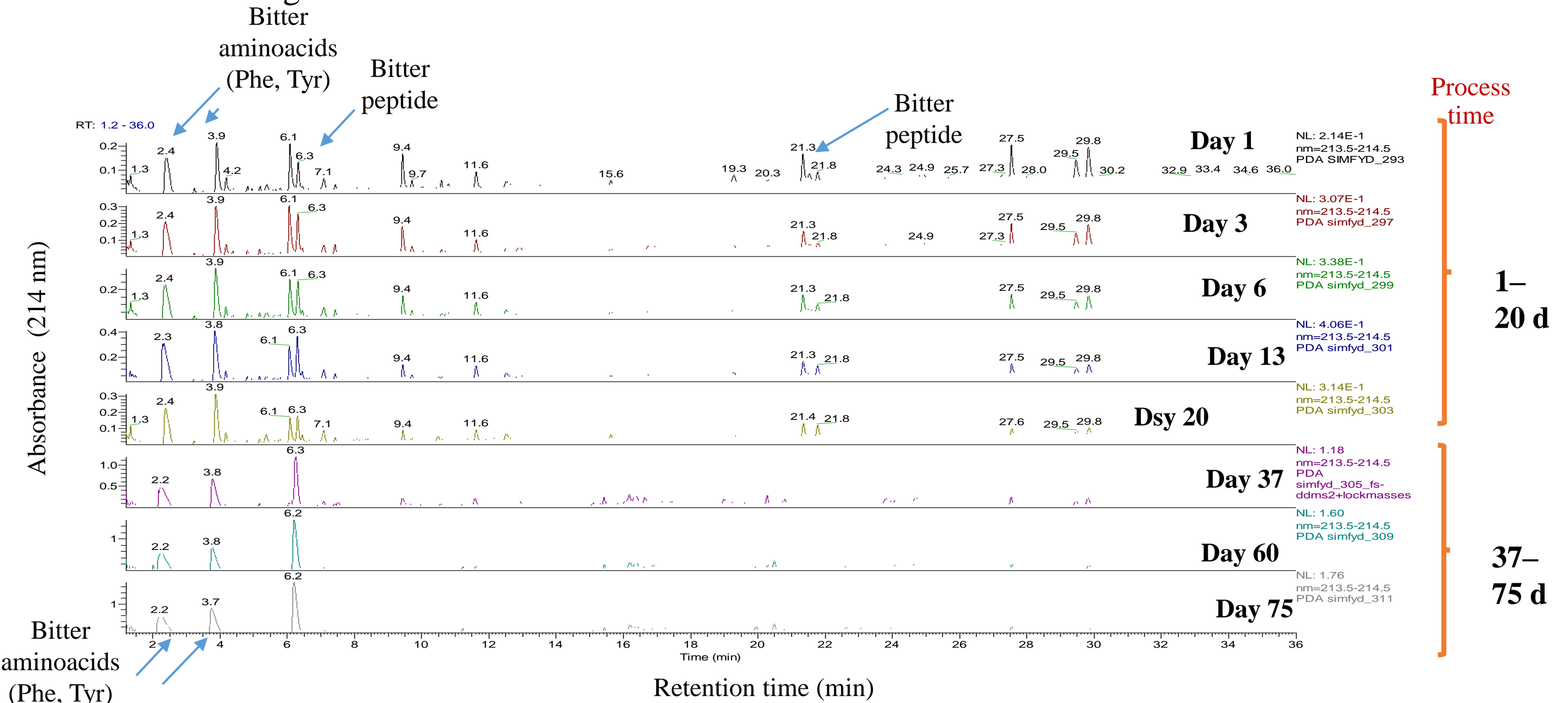

Figure 6: Mass Spectrometry plot describing the peptidic profile of Gongorzola samples.

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].

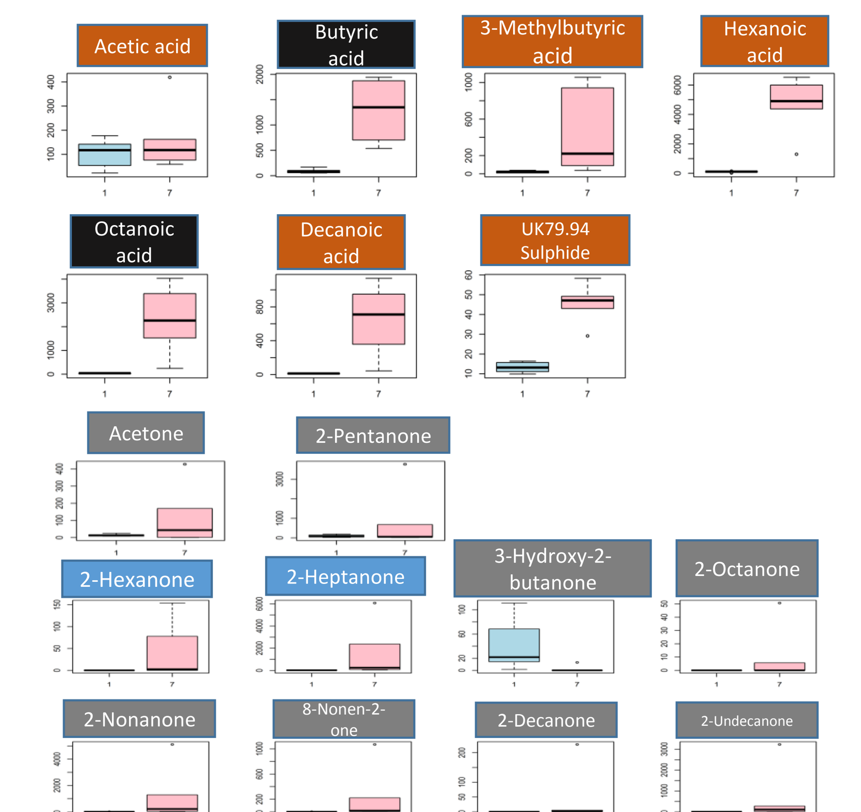

Figure 7: Box Plot representing the relative abundance (Arbitrary Units) of flavour-related Volatile Organic Compounds in the two key-phases of ripening. Quantitative data obtained with SPME-MS analysis on paste samples. **1** = non-mature samples (from day 1 to day 20); **7** = mature samples (from 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 «caves»; 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 *16S rRNA* 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.

References.
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[2] **Cocolin L., Nucera D., Alessandria V., Rantsiou K., Dolci P., Grassi M.A., Lomonaco S. and Civera T.** (2009) Microbial ecology of Gorgonzola rinds and occurrence of different biotypes of *Listeria monocytogenes*. *International Journal of Food Microbiology*, **133**, 200-205.
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