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 during ripening and in this project we found it in a brand new qRT-PCR approach, using a novel primer set directed against Aristilochene 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 37, before which the melting signals are very soiled and overlapped [Figure 1a]. In our hypothesis, the qPCR signal related to *P. roqueforti* was achieved after Day 37 [Figure 1b] as a consequence of a stronger increase of *P. roqueforti* micelia in cheese, whereas before Day 37 the amount of *P. roqueforti* micelia is too low and below the qPCR detection limit of 5 ng.

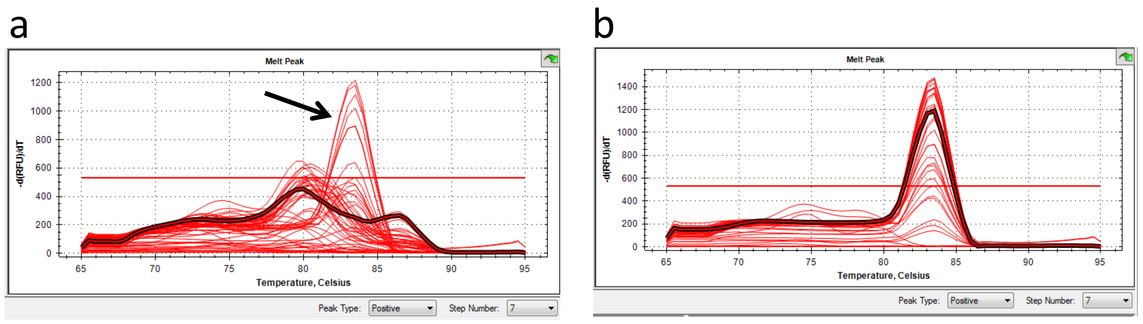


Figure 1: Melting curves from qPCR quantifications on Gorgonzola samples with Ari1 primer set; **a)** soiled samples from ripening Day 1 to Day 20: the melting peaks identied by the arrow belong to standard curve samples; **b)** clean samples from Day 37 to the end of ripening.

Signal "cleaning" 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 [Figure 2a] before Day 37. This allowed the correct quantification of the fungal mycelium during the whole ripening time [Figure 2b].

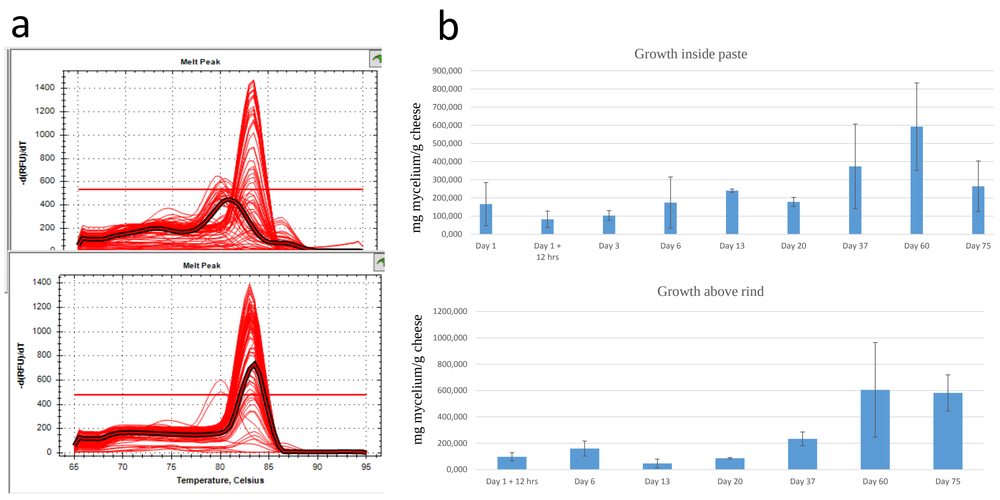


Figure 2: **a)** Comparison between melting profiles of Gorgonzola sample without (top) and with (bottom) 5 ng of pure exogenous *P. roqueforti* DNA added in reaction; **b)** quantifications of mycelium in paste and rind samples.

UPLC-MS analysis on peptidic profile. The Mass Spectrometric analysis revealed a visible simplification of the peptidic profile from the first phase (Day 1 to Day 20) until the second one associated to a strong *P. roqueforti* growth (Day 37 to Day 75) [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.

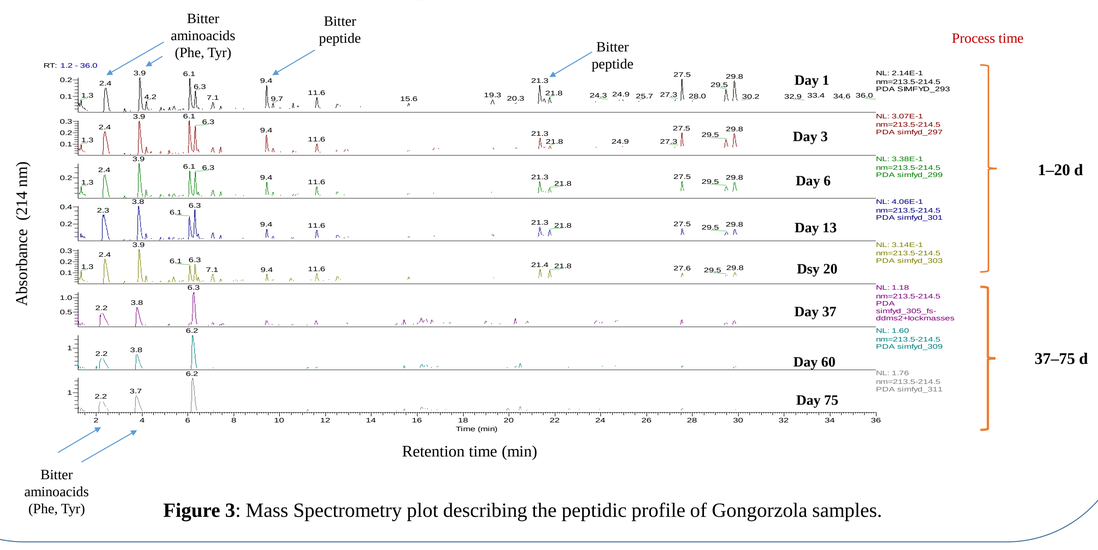


Figure 3: Mass Spectrometry plot describing the peptidic profile of Gongorzola samples.

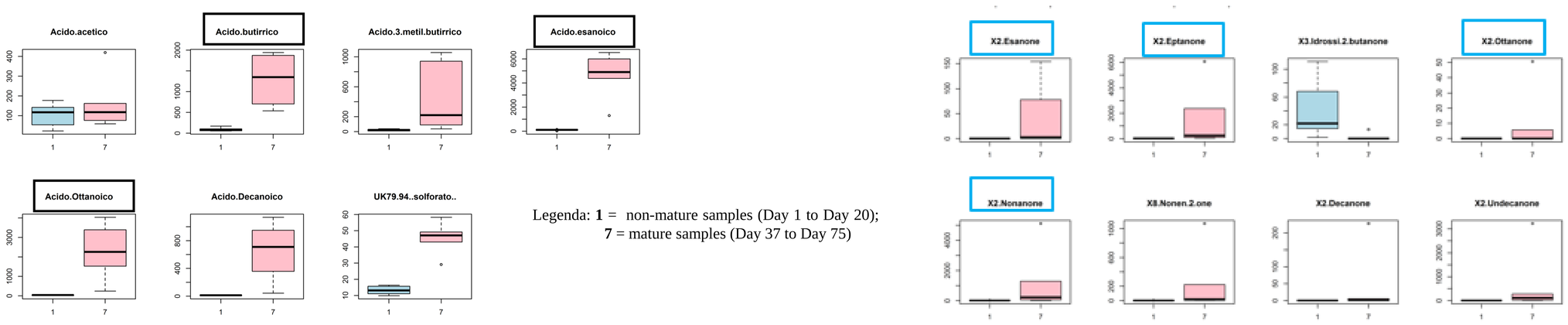
Identification of ACE-Inhibitor peptides. In the transition between the two phases, a remarkable increase in the quantity of Angiotensin Converting Enzyme-Inhibitor peptides, due to the intense proteolytic action of *P. roqueforti*, was observed [Table 1]. These peptides have a great biological value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

Sampling time-point	VPP	IPP	RYLG	RYLGY	AYFYPE	HLPLP	AYFYPEL	LHLPLP	Total
(d)					(mg kg^{-1})				
T1 (1)	0.73±0.13	0.24±0.02	0.05±0.01	nd	nd	0.15±0.02	nd	0.92±0.03	2.09±0.21
T2 (1.5)	1.20±0.37	0.30±0.04	0.06±0.01	nd	nd	0.19±0.02	nd	0.95±0.03	2.69±0.48
T3 (3)	1.88±0.19	0.35±0.02	0.07±0.01	nd	nd	0.19±0.03	nd	0.97±0.04	3.45±0.29
T4 (6)	1.20±0.01	0.29±0.00	0.12±0.01	nd	nd	0.31±0.00	nd	1.07±0.04	2.99±0.07
T5 (13)	0.94±0.32	0.29±0.06	0.15±0.01	nd	nd	0.54±0.10	nd	1.44±0.16	3.36±0.65
T6 (20)	0.40±0.02	0.17±0.00	0.19±0.02	nd	nd	0.64±0.18	nd	1.38±0.18	2.79±0.41
T7 (37)	3.92±1.24	11.74±2.19	0.31±0.02	nd	0.50±0.23	4.47±0.22	nd	4.95±0.19	25.89±4.10
T8 (60–62)*	9.16±0.65	28.05±1.44	0.06±0.01	nd	0.43±0.03	2.02±0.16	2.02±0.01	5.49±0.21	47.22±2.50
T9 (75)	9.06±2.73	25.74±4.66	0.08±0.02	nd	0.58±0.09	0.98±0.04	2.13±0.06	3.61±0.39	42.18±7.99

Cheese ripening age is expressed in days and indicated in brackets.
Values are presented as mean of two samples from paste ± 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 (Day 1 to Day 20) to the *P. roqueforti*-related phase (Day 37 to Day 75) is linked to the relative abundance of some important taste and odour descriptors increase [Fig.4], with particular attention to **hexanone** and **heptanone**, which are associated to typical ***"cheesy"*** flavour, and to **octanoic** and **butanoic** acid which are typically associated to ***putrid*** and ***rancid*** odour and fundamental for the organoleptic properties of Blue cheeses [Moio *et al.*, 2000].



Legenda: **1** = non-mature samples (Day 1 to Day 20);
7 = mature samples (Day 37 to Day 75)

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 Adsorption GC-MS analysis.

Conclusions. The analysis performed with qPCR molecular approach showed a great increase of *Penicillium roqueforti* mycelium growth from Day 37. The presence of a key phase in the maturation between Day 20 and Day 37 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.

References:

Dong, Y., Steffenson, B.J., Mirocha, C.J. (2006) Analysis of ergosterol in single kernel and ground grain by gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry* **54**, 4121-4125.
Moio, L., Addeo, F., Piombino, P. (2000) Odour-impact compounds in Gorgonzola cheese, *Journal of Dairy Research*, **67**, 273-285.
Proctor, R.H., Hohn, T.M. (1993) Aristolochene synthase. Isolation, characterization and bacterial expression of a sesquiterpenoid biosynthetic gene (Ari1) from *Penicillium roqueforti*. *The Journal of Biological Chemistry* **268**, 4543-4548.

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