BMMIB-Behind Microbe-Microbe Interactions in Blue Cheese

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The first two activities of this Ph.D. project are shown. Firstly, a deep analysis on Gorgonzola samples taken from diverse stage of the maturation was carried out, in order to provide a full microbiological and biochemical picture of the cheese in some key-moments of ripening. Secondly, a DNA extraction method was developed and a novel primer set was designed with the goal of establishing a very specific qPCR-based method to directly follow mycelial growth of *Penicillium roqueforti* during production process. Lastly, microbial dynamics in some key-phases were followed with a metagenomic approach.

Interazioni Microbiche in Formaggi Erborinati

Le prime due attività di questo progetto di Dottorato di Ricerca sono descritte. In primo luogo, è stata effettuata una profonda analisi di campioni di Gorgonzola presi da diverse fasi della maturazione, con lo scopo di fornire un quadro microbiologico e biochimico completo sul formaggio in diversi punti chiave della stagionatura. In secondo luogo, è stato sviluppato un metodo di estrazione di DNA e disegnato un nuovo primer set con l'obiettivo di costituire un nuovo metodo basato su qPCR specifica per seguire in tempo reale la crescita del micelio di *Penicillium roqueforti* durante il processo di stagionatura. In ultima analisi, le dinamiche microbiche in alcune fasi chiave della maturazione sono state seguite mediante un approccio metagenomico.

Key words: Lactic Acid Bacteria, Lipolysis, Metagenomics, *Penicillium roqueforti*, Proteolysis, qPCR.

1. Introduction

In accordance with the Ph.D. project previously described, this poster reports the results of the second and third milestones, concerning:
A1) the sampling and chemical/microbiological analysis on milk and Gorgonzola matrixes at each stage of production; the development of a metagenomic approach in order to follow the populations growth in cheese's paste and rind.
A2) the setting up of DNA extraction and the design and evaluation of a primer set species-specific for *Penicillium roqueforti* in order to develop a quantitative-PCR assay useful to follow the fungal biomass growth during cheese production.

2. Materials and Methods

The Gorgonzola samples were taken at each phase of ripening with sterile steel probing scalpels. The homogeneization of samples occurred with a StarBlender (Biosystems, Milan, Italy), using sterile MilliQ water to dilute samples 1:3. The samples underwent dilution and plating on diverse selective media (M17 for Streptococci, MRS for Lactobacilli and YGC for yeasts and moulds selection), and on each sample two types of chemical analysis were carried out: spectrophotometric analysis using enzymatic kits (R-Biopharm Italia, Melegnano, Italy) and deeper analysis on proteolysis and Volatile Organic Compounds using capillary electrophoresis and GC-MS methods. The metagenomics analysis were performed by an Illumina HiSeq 2500 sequencer (Illumina Inc., San Diego, CA, USA), and the sequencer data were processed with QIIME sequence-analysis software (www.qiime.com). The DNA extraction protocol was modified starting from a PowerFood DNA Extraction Kit (MoBio, Carlsbad, CA, USA) original protocol; in particular, in the initial phase, a thermal shock step (85°C for 30', -20°C for 30', repeated 3 times) and an enzymatical lysis step (Lyticase, 400 U/ml, 40°C for 1h, followed by Proteinase K, 1 mg/ml, 55°C, overnight) were added.
The primer set design started from the previously available sequence of Aristolochene synthase gene from *Penicillium roqueforti* (Proctor & Hohn, 1993), and the synthesis was performed by Primm Biotech (Milan, Italy). All qPCR evaluations and quantifications were carried out using a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA).

3. Results and Discussion

The microbiological data were collected with a classic dilution and plating approach on different selective media previously described in materials and methods. The numbers obtained on plate demonstrated the large growth of Starter Lactic Acid Bacteria until the first acidification of the pasteurized milk with the rennet, with a major stability of *Streptococcus thermophilus* in comparison with *Lactobacillus bulgaricus subsp. Delbrueckii* for the whole ripening time, due to the major oxygen-tolerance of Streptococci. In the same time, *Saccharomyces*

cerevisiae growth was already remarkable before the wheels entering the 22°C cabin: this early growth was linked to high temperature and the presence of substrates that derived from Lactic Acid Bacteria metabolism, as galactose.
All these data were later integrated with metagenomics analysis performed with Illumina system. The metagenomics 16S *rRNA* data revealed a relatively simple microbiological profile of the cheese, dominated by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, along with *Cellulosimicrobium cellulans*, which is previously known to be present expecially on rinds of Blue cheeses (Gobbetti *et al.*, 1997). *Micrococcus* species are also present, in a minor amount.
The metagenomics 18S *rRNA* data for eukaryotes presence revealed a microbiome dominated by *Saccharomyces cerevisiae*, with a sensible increase of the presence of *Debaryomyces hansenii* during ripening, expecially on rinds, as previously reported (Gobbetti *et al.*, 1997). The presence of *P. roqueforti* was detectable too.
In the next sampling more selective media will be used to establish if the microbial populations found in metagenomics analysis are cultivable on plate in the various phases of ripening, trying to address their role in the microbial dynamics together with already analysed starter cultures.
The dynamics clarified by microbiological data were integrated by an enzymatic spectrophotometric analysis on key metabolites. That revealed a massive production of ethanol – ascribable to *S. cerevisiae* growth – in the first phases of acidification, with a peak of 8 g/L reached inside the cabin, with the simultaneous consumption of galactose. Furthermore, a first evidence of the start of *Penicillium roqueforti* growth and proteolytic activity could be revealed by the enzymatic analysis on free ammonia production – with a great increase in the concentration of NH_4 following the increase of ripening days – and on lactic acid consumption – produced by Lactic Acid Bacteria, it's the most attractive substrate for the mould. The evaluation of proteolysis and lipolysis during ripening, was evaluated with a GC-MS analysis, and disclosed the presence of a key-phase, between sampling at Day 20 and sampling at Day 37 in which *P. roqueforti* growth withstand a great increase, revealed both by the simplification of the peptidic profile – with the disappearance of bitter peptides –and a remarkable change in the Volatile Organic Compounds profile. In particular, in the transition between one phase to another, the relative abundance of some important taste and odour descriptors had an increase, with particular attention to Hexanone and Heptanone – typical "cheesy" flavour – or to Octanoic and Butanoic Acid – typically associated to putrid and rancid odour – that were described to be foundamental for the organoleptic properties of Blue cheeses (Moio *et al.*, 2000).
The evaluation of a novel primer set to develop a qPCR assay to monitor in real-time during ripening the maturation of *P. roqueforti* mycelium – which is fundamental for the quality of the finished product – was carried on. The primer set was designed on an arbitrary chosen biosynthetic gene, Aristolochene synthase – ARI1, involved in the production of PR mycotoxin – from *P. roqueforti*, previously isolated and sequenced (Proctor & Hohn, 1993). After a qPCR trial carried on with a temperature gradient on plate, to find out the best annealing temperature for the primer set – established at 52,4 °C – another qPCR trial was performed in order to evaluate the specificity of ARI1 for *P. roqueforti*, using as competitor a previously known primer set directed on *P. roqueforti* 18s *rRNA*, named ITSRoq1 (Le Drèan *et al.*, 2009). Pure DNA from *P. roqueforti*, together with pure DNA extracted from other phylogenetically similar fungi – in particular *P. chrysogenum* and *P. camemberti* – and from common contaminants of blue cheese production plants – like *Geotrichum candidum* – was used as target. The analysis revealed that ARI1 was able to amplify with good efficiency only *P. roqueforti* DNA, thus the primer set revealed to be more specific than the previous ones known in literature, and was chosen to quantify mycelium mass during the various ripening phases. As previously unravelled by chemical analysis, also in molecular trials a key-change was revealed in the passage between Day 20 and Day 37: the qPCR profile before Day 37 resulted very soiled, with a series of unknown peaks overlapping with ARI1; instead, after that passage, the profile resulted completely clean, with the clear presence of the peak related to ARI1 signal. This sudden changing in the signal properties was found out also with analysis performed with the other primer set available, ITSRoq1. In order to correctly quantify the mycelium in soiled samples belonging to phases prior to Day 20, a cleaning method for ARI1 signal was evaluated. 5 ng of pure *P. roqueforti* DNA was added to each reaction mix, and the final data resulted completely clean for every sample. The final quantification of *P. roqueforti* mycelium was then normalized by subtraction of the known amount of mycelium corresponding to the 5 ng of pure DNA added; the difference of quantification between clean samples and clean samples added with pure DNA was considered statistically non-significant. The quantifications revealed a strong growth of *P. roqueforti* in the phase after Day 37, reaching up to 800 mg mycelium/g cheese. In the next phases of the Ph.D. project, the potentiality of this cleaning approach for an on-line quantification of *P. roqueforti* mycelium during production process will be evaluated with closer sampling to deeper investigate this pivotal phase between Day 20 and Day 37.

4. References

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