

## **Detection and evaluation of viability and activity of microorganisms in food by using culture independent approaches**

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This PhD thesis research project is aimed to investigate microbial population in complex ecosystems such as fermented foods by using culture independent approaches. In this context already adopted techniques, such as LH-PCR and FISH will be strengthened; moreover enumeration of total viable microorganisms present in fermented dairy products by using a novel multiplex quantitative reverse-transcription PCR (qRT-PCR) analysis will be performed. A multiplex strategy to simultaneously quantify different lactic acid bacteria in a heterogeneous microflora will be considered.

### **Valutazione della presenza, vitalità e attività dei microrganismi negli alimenti mediante tecniche di non coltivabilità**

Questo progetto di tesi di dottorato ha lo scopo di valutare la popolazione microbica in ecosistemi complessi quali gli alimenti fermentati sfruttando un approccio culture independent. In questo contesto saranno migliorate tecniche già adottate, come la FISH e la LH-PCR; inoltre saranno quantificati i microrganismi vitali totali presenti in prodotti lattiero-caseari fermentati utilizzando un'innovativa analisi quantitativa mediante retroscrittione-PCR multiplex. Sarà considerata una strategia multiplex per quantificare simultaneamente differenti specie di batteri lattici all'interno di una microflora eterogenea.

#### **1. State-of-the-Art**

Foods are complex ecosystems composed of the environment and the organisms that live in it. Microbial population in foods, in particular fermented foods, are normally constituted by many different species that have markedly different growth requirements and come from different sources. As microorganisms are extremely diverse and highly dynamic, it is not surprising that the many different types of microbial species present in food exist in a number of physiological states that possess different requirements for survival and growth (Dolan et al., 2009).

Culture-based techniques have been widely used to study these complex ecosystems, however, there are many drawbacks. Understanding the dynamics of such communities is very laborious and time-consuming and the inability to cultivate more than a small proportion of the microorganisms that can be visualized by direct count procedures makes it difficult to obtain information on the entire microbial community (Jany et al., 2008). Moreover, under some stress environmental conditions cells are induced to a physiological state in which they are metabolically active but incapable to form colonies in plate cultures, a state so called "viable but non cultivable". Furthermore, species occurring in low numbers are often out-competed *in vitro* by numerically more abundant microbial species (Hugenholz et al., 1998).

Thus, to study interactions among microorganisms, it is important to study the ecosystem without dissociating it. Culture-independent techniques, such as FISH (fluorescent *in situ* hybridization) using rRNA-targeted oligonucleotide probes (Ercolini et al., 2003; Bottari et al., 2006) and those involving PCR, provide new insight by helping to define the biodiversity of communities and allowing the detection and identification of viable, non viable, and viable but non cultivable populations. Various PCR-based molecular typing methods have been developed for the analysis of populations, providing a rapid, yet well representative fingerprint of the microbial community structure without cultivation (Ercolini et al., 2002; Duthoit et al., 2003; Hori et al., 2006). An increasing number of new methodologies employ automatic sequencing systems with laser detection of fluorescently labelled DNA fragments (Marsh 1999; Osborn et al., 2000; Sanchez et al., 2006; Suzuki et al., 1998). Length heterogeneity PCR (LH-PCR) analysis distinguishes different organisms based on natural variations in the length of 16S ribosomal DNA sequences. To date it has been used to study microbial communities in different environments (Ritchie et al., 2000; Brusetti et al., 2006) and in food-associated ecosystems such as dairy (Lazzi et al., 2004).

In a complex ecosystem, not only identification but also quantification is important to obtain the real composition and the relative proportions of microbial species. Recently, real-time PCR has demonstrated to be a powerful technique to reach this goal in several food matrices (Friedrich and Lenke, 2006; Pfannebecker and Fröhlich, 2008; Scheirlinck et al., 2008; Sheu et al., 2009). A disadvantage of DNA-based methods is that they do not distinguish between viable and non-viable cells. DNA from lysed cells can persist for a long time in the

environment; detection of RNA would help to determine the viability of the microorganisms because it is degraded rapidly upon cell death. Coupling reverse transcription with real time quantitative PCR (qRT-PCR) for enumeration of microorganisms has been shown to be both accurate and sensitive for quantification purposes (Hierro et al., 2006) and it has the potential to be applied directly to enumerate viable microorganisms in food samples as an alternative approach with respect to the standard plate and direct count methods (Dolan et al., 2009).

## 2. 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:

A1) **Assessment of complex microbial populations by using LH-PCR technique** in natural whey starters and long ripened cheese, improvement of the method to better discriminate microorganisms and a deeper knowledge of unknown data registered so far;

A2) **Improvement of FISH analysis** and coupling with LH-PCR technique for a polyphasic approach on solid dairy matrices;

A3) **Development of a novel multiplex qRT-PCR strategy** to simultaneously quantify different lactic acid bacteria in dairy matrices: choice of the target gene (A3.1), primer design (A3.2), assessment in a dairy model with known bacterial species (A3.3), setting up an efficient RNA extraction method from dairy matrices (A3.4), application of the novel multiplex qRT-PCR to enumerate microbial composition in natural whey starters (A3.5);

A4) **Assessment of microbial population dynamics during hard cheeses fermentation and ripening.** Long ripened cheeses (such as Parmigiano Reggiano and Grana Padano) will be collected at different stages of ripening and will be analyzed by using culture independent techniques;

A5) **Writing and Editing of the PhD thesis**, scientific papers and oral and/or poster communications.

**Table 1:** Gantt diagram for this PhD thesis project.

Activity		Months																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	<b>LH-PCR improvement</b>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A2)	<b>FISH improvement</b>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A3)	<b>Multiplex qRT-PCR</b>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	1) Choice of target gene	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	2) Primer design	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	3) Assessment in a dairy model	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	4) RNA extraction method	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	5) Whey starter analysis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A4)	Long ripened cheese sampling and analysis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A5)	Thesis and Paper Preparation	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

## 3. Selected References

- Bottari B, Ercolini D, Gatti M, Neviani E (2006) Application of FISH technology for microbiological analysis: current state and prospects. *Appl Microbiol Biotechnol* **73**:485-94.
- Brusetti L, Borin S, Mora D, Rizzi A, Raddadi N, Sorlini C, Daffonchio D (2006) Usefulness of length heterogeneity-PCR for monitoring lactic acid bacteria succession during maize ensiling. *FEMS Microbiol Ecol* **56**:154-164.
- Dolan A, Burgess CM, Barry TB, Fanning S, Duffy G (2009) A novel quantitative reverse-transcription PCR (qRT-PCR) for the enumeration of total bacteria, using meat micro-flora as a model. *J Microbiol Methods* **77**:1-7.
- Duthoit F, Godon JJ, Montel MC (2003) Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl Environ Microbiol* **69**:3840-48.
- Ercolini D, Blaiotta G, Moschetti G, Coppola S (2002) Evaluation of PCR-DGGE analysis for molecular typing of cheeses. *Ann Microbiol* **52**:81-7.
- Ercolini D, Hill PJ, Dodd CER (2003) Development of a fluorescence in situ hybridization method for cheese using a 16S rRNA probe. *J Microbiol Methods* **50**:97-100.

- Friedrich U, Lenke J (2006) Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization. *Appl Environ Microbiol* **72**:4163-71.
- Hierro N, Esteve-Zarzoso B, González A, Mas A, Guillamón JM (2006) Real-time quantitative PCR (qPCR) and reverse transcription-qPCR for detection and enumeration of total yeasts in wine. *Appl Environ Microbiol* **72**:7148-55.
- Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y (2006) Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16S rRNA gene fragments. *J Microbiol Methods* **66**:165-69.
- Hugenholtz P, Goebel BM, Pace NR (1998) Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *J Bacteriol* **180**:4765-74.
- Jany JL, Barbier G (2008) Culture independent methods for identifying microbial communities in cheese. *Food Microbiol* **25**:839-48.
- Lazzi C, Rossetti L, Zago M, Neviani E, Giraffa G (2004) Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. *J Appl Microbiol* **96**:481-90.
- Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* **2**:323-27.
- Osborn AM, Moore ERB, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community and dynamics. *Environ Microbiol* **2**:39-50.
- Pfannebecker J, Fröhlich J (2008) Use of a species-specific multiplex PCR for the identification of pediococci. *Int J Food Microbiol* **128**:288-96.
- Ritchie NJ, Schutter ME, Dick RP, Myrold DD (2000) Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. *Appl Environ Microbiol* **66**:1668-75.
- Sanchez JI, Rossetti L, Martinez B, Rodriguez A, Giraffa G (2006) Application of reverse transcriptase PCR-based T-RFLP to perform semi-quantitative analysis of metabolically active bacteria in dairy fermentations. *J Microbiol Methods* **65**:268-77.
- Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, Vandamme P, Huys G (2008) Taxonomic structure and stability of the bacterial community in belgian sourdough ecosystems as assessed by culture and population fingerprinting. *Appl Environ Microbiol* **74**:2414-23.
- Sheu SJ, Hwang WZ, Chen HC, Chiang YC, Tsen HY (2009) Development and use of tuf gene-based primers for the multiplex PCR detection of *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Lactobacillus delbrueckii*, and *Bifidobacterium longum* in commercial dairy products. *J Food Prot* **72**:93-100.
- Suzuki MT, Rappe MS, Giovannoni SJ (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Appl Environ Microbiol* **64**:4522-29.