Università degli Studi di Parma

Faculty of Agriculture

Ph.D. in Food Science and Technology

XXI° cicle 2006-2008

Ph.D. dissertation

Culture independent approach for the evaluation of presence and activity of microorganisms in food

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Parma 2009

To my Family, Friends and Professors

"Whatever you can do, or dream you can, begin it. Boldness has genius, power, and magic in it. Daring ideas are like chessmen moved forward; they may be beaten, but they may start a winning game".

Johann Wolfgang von Goethe

1. Introduction

1.1 Microbial communities in foods

Food are complex ecosystems, composed of the environment and the organisms that live in it. Microbial population in foods, food ingredients, and the food contact environment, is normally constituted by many different species that have markedly varied 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 to sustain growth (Dolan et al., 2008). Discussion of the role of microorganisms in food processing is often confined to negative aspects, such as their roles in spoilage and in food-borne illness, but the almost infinite number of combinations of microbe and foodstuff (Adams 1990), gave human the way to help preserve or transform foods since millenia. The interest in the application of microorganisms and their metabolites in the prevention of the food spoilage and the extension of the shelf life of foods have been increased during the years (Stiles et al., 1996). Further, many fermented food and beverage evolved as a means of food preservation, became appreciated for desirable flavour, aroma and texture given by microorganisms. Being able to control the specific microorganisms or the succession of microorganisms that dominate the microflora of foods (which is the basis of development of starter cultures) is of key importance for maintaining quality and safety of foods (Caplice et al., 1999). The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to the changing of the physical and chemical conditions into the food micro-environment (e.g., pH, salt, temperature), the ability to colonise

the food matrix and to growth into a spatial heterogeneity (e.g., micro-colonies and bio-films), and the in situ cell-to-cell interactions which often happen in a solid phase (Giraffa and Neviani 2001). Microbial growth in foods is thus a complex process governed by genetic, biochemical and environmental factors. Much of what we know about foodborne microbes must be held with the detached objectivity required of an unproven hypothesis (Montville and Matthews, 2001). Developments in studying microbial ecology are continuously changing and deepening our perspective about the relation between food and microorganisms. This way, culture-independent methods are getting more and more used.

1.2 Towards culture independent methods

The growth, survival, and activity of any microbial species present in a food, being an unwanted spoilage or pathogenic organism, or a desirable biocontrol of a probiotic agent, will be, in most cases, determined by the presence of other microorganisms and by the in situ cell-to-cell ecological interactions between microorganisms and food. Understanding the patterns of occurrence of microbial populations in food is really a problem of determining the actual niche of the organism present. That is, identifying the location (in terms of resource quality, microclimate, and presence of other microorganisms) and the role of an organism in both space and time. Reliable quantitative ecological data should, therefore, take into consideration the dynamics of microorganisms in food ecosystems. This information is of key importance in food ecology, to understand the behaviour of all those microbial species involved in foods production, storage, quality and safety. A first step toward introducing ecological concepts in food microbiology is to understand microbiological profiles and microbial community structures, dynamics, and functioning in foods in relation

to changing of environmental determinants (the “habitat domain”) and biological conditions over space and time (the “activity domain”). Such objectives could be reached by the use of culture-independent methods (Giraffa and Neviani 2001). It is widely accepted that plate culturing techniques reveal a small portion (i.e., that viable and culturable) of the true microbial population in natural ecosystems. This can essentially be explained by two ecological factors: (i) an inability to detect novel microorganisms, which might not be cultivable with known media and (ii) an inability to recover known microorganisms which are either stressed or actively growing but which enter a non-cultivable state (Giraffa 2004). Traditional methods to determine the composition of microflora require cultivation on selective media, which is laborious, time-consuming, and prone to statistical and methodological errors (Moter and Gobel, 2000). According to classic microbiology in fact, to study a microorganism it is necessary to isolate it from the original matrix and this isolation can be done only on the opportune medium, from the plates showing separately grown-up colonies, that is to say from the plates corresponding to the so-called countable dilutions. Moreover, it has been demonstrated how any environmental modification during cultivation, could affect the microbial communities structure, preventing from obtaining a complete view of the considered ecosystem. Therefore, isolation and cultivation in agar medium allow to characterize only those microorganisms capable of growing, multiplying and forming colonies in the selected medium and condition of growth, loosing the information regarding the microbial component which is in a vitality condition even if it can’t duplicate in culture medium. These limits restrict and bias in an unpredictable way our view of microbial diversity (Giraffa and Neviani 2001). Even though new microrganisms are continuously isolated, it is estimated that only a small fraction of

the existent microorganisms has been grown in pure culture and characterized. The lack of knowledge is most severe for complex multi-species microbial communities. Even when all bacteria can ultimately be cultured (which is quite unlikely), progress in the understanding of the ecology of complex microbial communities require studies on the activity and distribution of microbes directly in minimally disturbed samples (Amann et al., 1998). Research on the food microflora, which has relied so far mainly on cultivation, may not always be representative of the complexity of a food ecosystem (Lazzi et al. 2004). Further, viability assessment of microorganisms is relevant for a wide variety of applications in food industry, thus, new ways to gain more knowledge on the physiological state (viability), the stress response, and the survival of microorganisms into foods, especially on those stressed, sub-lethally injured, or otherwise "viable but non-culturable" cells which often go undetected when using traditional microbiological techniques, are needed. To determine the diversity of different food associated microorganisms and to monitor evolution of microbial population over space or time, culture independents method such as DGGE/TGGE, fluorescence in situ hybridization, and PCR-based techniques such as reverse transcriptase-PCR (RT-PCR), terminal-restriction length polymorphism (T-FRLP), tDNA intergenic spacer-PCR (tDNA-PCR) and length heterogeneity PCR (LH-PCR) have been developed (Ercolini et al., 2001, 2003a; Rademaker et al., 2005, Ogier et al., 2002; Randazzo et al., 2002).

1.3 Viability assessment

Several cells physiological states may not allow the cultivation of cells on agar medium. Dead, sublethally damaged, viable but non-culturable, dormant, and inactive cells could then go undetected with traditional counting methods but they could still play a role in the ecosystem they are part of. Determination of cells viability, in term of

physiological activity, is then of key importance for studying the relation microorganisms-environment. In food ecosystems, the viability of microorganisms is crucial for applications such as detection and enumeration of food spoilage microorganisms, evaluation of inactivation treatments, quality assessment of starter cultures, biodegradation, production of antibiotics, and numerous others (Breeuwer et al. 2000). The elementary requirements for viable microorganisms to survive are: (i) an intact cyto-plasmic (plasma) membrane which functions as a barrier between the cytoplasm and the extracellular environment; (ii) DNA transcription, and RNA translation; (iii) generation of energy for maintenance of cell metabolism, biosynthesis of proteins, nucleic acids, polysaccharides, and other cell components; and, eventually, (iv) growth and multiplication. Methods for assessment of cell viability are based on these requirements (Breeuwer et al. 2000). Among them, fluorescent techniques coupled with flow cytometry or image analysis, can be used for a high sensitivity and rapid assessment of microorganisms viability (Breeuwer et al. 2000). Fluorescent staining have been successfully used for the evaluation of viability in several food matrices such as whey starters (Gatti et al 2006), probiotic dairy products (Auty et al., 2001) and beer (Boyd et al., 2003).

1.4 LH-PCR

LH-PCR is a high resolution electrophoretic method for the analysis of rDNA diversity. LH-PCR analysis allow to distinguish different organisms based on natural variations in the length of 16S rRNA gene (or other genes) sequences. Hyper-variable regions of the 16S rRNA gene, which are located at the 5'-end of the bacterial gene, are amplified using a fluorescently labelled oligonucleotide as forward primer, coupled with an unlabelled reverse primer (Giraffa and Neviani

2001). Natural length polymorphisms in the 5' region of the molecules are reflected in the lengths of DNA fragments of the PCR amplicon. Labelled fragments are separated by capillary (or gel) electrophoresis and detected by laser-induced fluorescence with an automated gene sequencer. A fluorescent internal size standard is run with each sample and is used to size the amplicon lengths in base pairs. The fluorescent data are converted into chromatographic profiles called electropherograms. The intensity (height) or area under the peak in the electropherogram is proportional to the relative abundance of that particular amplicon (Mills et al., 2007). The relative amounts of amplified sequences originating from different microorganisms can be then determined. LH-PCR does not need post-PCR clean up or restriction enzyme digestion and, if DNA extraction methods is robust, gives replicate profiles highly reproducible. Problems could arise for profiles showing contiguous amplicon distributions that are sometimes difficult to resolve. Further, one amplicon can represent more than one taxon that are philogenetically distinct but produce the same amplicon (Mills et al., 2001). LH-PCR has been successfully used to study complex ecosystems such as natural whey starter for Grana Padano cheese (Lazzi et al., 2004; Santarelli et al 2008), Parmigiano Reggiano cheese microflora (Gatti et al., 2008), maize silage (Brusetti et al., 2006), soil (Ritchie et al., 2000) and plankton (Suzuki et al., 1998).

1.5 FISH

Among the molecular approaches that enable cultivation-independent characterization of microorganisms, fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes (Amann et al., 1995, DeLong et al., 1989) has been one of the most powerful and widely used techniques (Amann et al., 2001) in microbial ecology, thus providing microbial identification, physical detection of uncultivable

microorganisms, and distribution of microbial populations in several environments, including food products. FISH has been used to evaluate bacterial community structure and location in Stilton cheese (Ercolini et al., 2003a) and (Ercolini et al., 2003b), to detect *Brevibacteria* on the surface of Gruyère cheese (Kolloffel et al., 1999), to detect *Lactobacillus plantarum* on natural fermented olives (Ercolini et al., 2006), and to quantify *Leuconostoc* populations in mixed dairy starter cultures (Olsen et al., 2007).

1.5.1 Application of FISH technology for microbiological analysis: current state and prospects

Benedetta Bottari, Danilo Ercolini, Monica Gatti, Erasmo Neviani.
Application of FISH technology for microbiological analysis: current state and prospects. Appl Microbiol Biotechnol 2006, 73(3):485-94

1.5.1: 1 Abstract

In order to identify and quantify the microorganisms present in a certain ecosystem, it has become necessary to develop molecular methods avoiding cultivation, which allow to characterize only the countable part of the microorganisms in the sample, therefore losing the information related to the microbial component which presents a vitality condition, although it cannot duplicate in culture medium. In this context, one of the most used techniques is fluorescence in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes. Owing to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology. Through the use of species-specific probes, it is possible to identify different microorganisms in complex microbial communities, thus providing a solid support to the understanding of interspecies interaction. The knowledge of the composition and distribution of microorganisms in natural habitats can be interesting for ecological reasons in microbial ecology, and for safety and technological aspects in food microbiology. Methodological aspects, use of different probes and applications of FISH to microbial ecosystems are presented in this review.

1.5.1: 2 Introduction

The lack of morphological details in many bacteria and microorganisms has usually hampered their identification, making the use of cultivation necessary for identification. This has proven difficult

for many environmental or medically important microorganisms (Amann and Kuhl 1998). Traditional methods to determine the composition of microflora require cultivation on selective media, which is laborious, time-consuming and prone to statistical and methodological errors (Moter and Gobel 2000). According to classical microbiology, in order to study a microorganism, it is necessary to isolate it from the original matrix, and this isolation can be done only on the appropriate medium, from the plates showing separately grown colonies, i.e. from the plates corresponding to the so-called countable dilutions. Moreover, it has been demonstrated how any environmental modification during cultivation could affect the structure of microbial communities, thus preventing a complete view of the ecosystem considered. Therefore, isolation and cultivation in agar medium allow to characterize only those microorganisms capable of growing, multiplying and forming colonies in the selected medium and condition of growth, with the loss of the information relative to the microbial component which presents a vitality condition despite not being able to duplicate in culture medium. Although new microorganisms are continuously isolated, it is estimated that only a small fraction of the existent microorganisms have been grown in pure culture and characterized. The lack of knowledge is most severe for complex multi-species microbial communities. Even when all bacteria could ultimately be cultured (which is quite unlikely), progress in the understanding of the ecology of complex microbial communities would require studies on the activity and distribution of microbes directly in minimally disturbed samples (Amann et al. 1998).

Therefore, in order to identify and quantify the microrganisms occurring in a certain ecosystem, the development of molecular methods avoiding cultivation has become necessary. In this contest, one of most used techniques is fluorescence *in situ* hybridization

(FISH) with ribosomal RNA (rRNA) targeted oligonucleotide probes (Langedijk et al. 1995; Amann et al.1990). This method is based on the hybridization of synthetic oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation.

FISH originated in medicine and developmental biology for the localization of particular DNA sequences in mammalian chromosomes and subsequently has been applied primarily in environmental bacteriology (Amann et al. 1995) and to a lesser extent in protist ecology (Lim et al. 1996). Owing to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology.

FISH has so far been applied to the study of microbial symbiosis, and microbial diversity in environmental samples and wastewater treatment (Amann et al. 2001). It is also routinely used in medicine as a diagnostic tool for the identification of bacteria in complex communities colonising the oral cavity, the respiratory and gastro-intestinal tracts, as well as for the detection of pathogens within human and animal tissues (Moter and Gobel 2000).

FISH combines the precision of molecular genetics with the visual information from microscopy, allowing visualization and identification of individual cells within their natural microhabitat or diseased tissue, so that nucleic acid sequences can be examined inside cells without altering the cell's morphology or the integrity of its various compartment (Moter and Gobel 2000). FISH techniques for detecting RNAs have been introduced into living cells using either fluorophores that can be "un-caged" in vivo, or probes that fluoresce only when hybridized.

One drawback of live-cell *in situ* hybridization is that FISH requires mechanical disturbance of the cell in order to introduce probes. *In situ* identification of individual microbial cells with fluorescently labelled,

rRNA targeted oligonucleotide probes, the so called phylogenetic stains, is based on the high cellular content of ribosomes, which can be found in all living organisms, and consequently as many 16S and 23S rRNA molecules (Amann et al. 1998). rRNAs are the main target molecules even because they are relatively stable and they include both variable and highly conserved sequence domains (Amann et al. 2001). The selection of particular regions of the rRNA molecule enables then phylogenetic specificity to be varied from the universal to the subspecies level (DeLong et al. 1989; Amann et al. 1990), even if, because of the relatively slow mutation rate of rRNA, this molecule generally possesses no target sites that differentiate between strains of prokaryotic species (Wagner et al. 2003). Under appropriate reaction conditions, complementary sequences in the probe and target cell anneal, and the site of probe hybridization is detected by fluorescence microscopy (DeLong et al. 1989; Amann et al. 1990). FISH allowed significant advances in resolution, speed and safety, and later paved the way for the development of simultaneous detection of multiple targets, quantitative analysis and live-cell imaging (Levsky et al. 2003). These techniques allow deeper study of live gene expression in a minimally disturbed context, but must be interpreted taking into consideration the possible artefacts that may result as physiological ramification of hybridization (Levsky et al. 2003).

1.5.1: 3 Methodological aspects

FISH with rRNA target probes has been developed for the *in situ* identification of single microbial cells and is the most commonly applied among the "non-PCR-based" molecular techniques (Amann et al. 1990, 2001; Moter and Gobel 2000). A typical FISH protocol (Fig.1) includes four steps: fixation and permeabilization of the sample;

hybridization; washing steps to remove unbound probe; and detection of la belled cells by microscopy or flow cytometry (Amann et al. 2001).

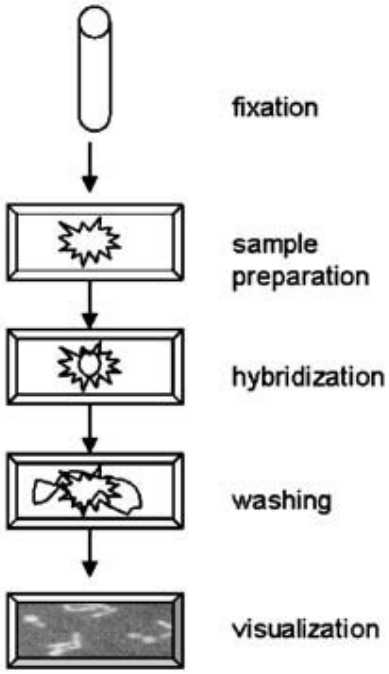

Fig.1 Flow chart of a typical FISH The procedure includes the following steps: (1) fixation of the specimen directly in sample tubes; (2) transfer of the sample on a glass slide and preparation of the sample, including specific pre-treatment steps; (3) hybridization with the respective probes for detecting the respective target sequences; (4) washing steps to remove unbound probes; (5) mounting, visualization and documentation of results.

Prior to hybridization, bacteria must be fixed and permeabilized in order to allow penetration of the fluorescent probes into the cell and protect the RNA from degradation by endogenous RNAs (Moter and Gobel 2000). The sample is either settled on membrane filters and covered with the fixing agent (Glockner et al. 1999), or mixed with the fixing agent, incubated, sedimented by centrifugation, resuspended, transferred to glass slides and dried (Amann et al. 1990). For better attachment of specimens to glass slides, some author suggested first treating the surfaces with coating agents such as gelatin (Amann et al. 1990), poly-L-lysine (Lee et al. 1999) or silanating agents (Moter et al. 1998). If cell suspensions are investigated, bacteria are fixed in suspension, spotted onto microscopic slides, air dried and dehydrated

in an ethanol series. In some cases, e.g. for Gram-positive cells, an additional enzymatic treatment with lysozyme (Schonhuber et al.1997; Wagner et al. 1998), lysostaphin, or an enzyme mixture (Krimmer et al. 1999) may be necessary to open the peptidoglycan layer.

Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. For this crucial step of the FISH procedure, a preheated hybridization buffer is applied to the sample containing fluorescently labelled probes complementary to the target RNA. The hybridization takes place in a dark humid chamber, usually at temperatures between 37°C and 50°C. Hybridization time varies between 30 min and several hours.

The slides are then briefly rinsed with distilled water to remove unbound probe. Finally, the slides are rinsed with water again, dried, possibly mounted in anti-fading agents to prevent fluorescence "bleaching" (Moter and Gobel 2000), then the results are visualized and documented.

The oligoucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked to a single fluorescent dye. Common fluorophores are reported in Table 1, and include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5 (Amann et al. 2001).

Fluorochrome	Colour	Max. excitation λ (nm)	Max. emission λ (nm)
Alexa488	Green	493	517
AMCA	Blue	399	446
CY3	Red	552	565
CY5	Red	649	670
CY7	Violet	743	767
DAPI	Blue	350	456
Fluorescein	Green	494	523
Rodamine	Red	555	580
TAMRA	Red	543	575
Texas red	Red	590	615
TRITC	Red-orange	550	580

Table 1 Most commonly employed fluorescent dyes to label oligonucleotides for FISH analysis. AMCA methyl cumarinic acetic acid, CY carbocyanine, DAPI 4′6-diamidino-2-phenylindole dihydrochloride, TAMRA tertramethylrhodamine, TRITC tetramethylrodamine-isotiocianate

The number of target cells detected, for example, with the universal oligonucleotide probe Eub338 ranges from 1% to 100% of the total bacterial count in enriched culture, even depending on the physiological state of the cells (Bouvier and Del Giorgio 2003). This wide range implies that FISH is extremely sensitive not only to variations in the methodological aspects of the protocol but even to environmental conditions. De Vries et al. (2004) observed that the growth phase influenced both the amount of the rRNA present per cell and the fluorescence intensity of FISH experiments. They noticed that even if the amount of rRNA is maximum at the start of the stationary growth phase as reflection of adaptation to new conditions representative for the growth phase, FISH can be used to estimate the *in situ* activity of *Lactobacillus plantarum* only during the first exponential growth phase. These contrasting results are probably due to changes taking place in the cell envelope during the second exponential growth phase which prepare cells for survival under

stationary growth phase and which cause difficulties in the permeabilization of the cells.

1.5.1: 4 Probes labelling

The earliest *in situ* hybridizations, performed in the late 1960s, were not fluorescent at all, but rather utilized probes labelled with radioisotopes. The first application of fluorescent *in situ* detection came in 1980, when RNA was directly labelled on the 3' end with a fluorescent molecule (Bauman et al. 1980). Enzymatic incorporation of fluorophore-modified bases throughout the length of the probe has been widely used for the preparation of fluorescent probes (Wiegant et al. 1991). In the early 1990s, improved labelling of synthetic, single-stranded DNA probes allowed the chemical preparation of hybridization probes carrying enough fluorescent molecules to allow direct detection (Kislauskis et al. 1993). Nowadays, there are different ways of labelling (Fig 2).

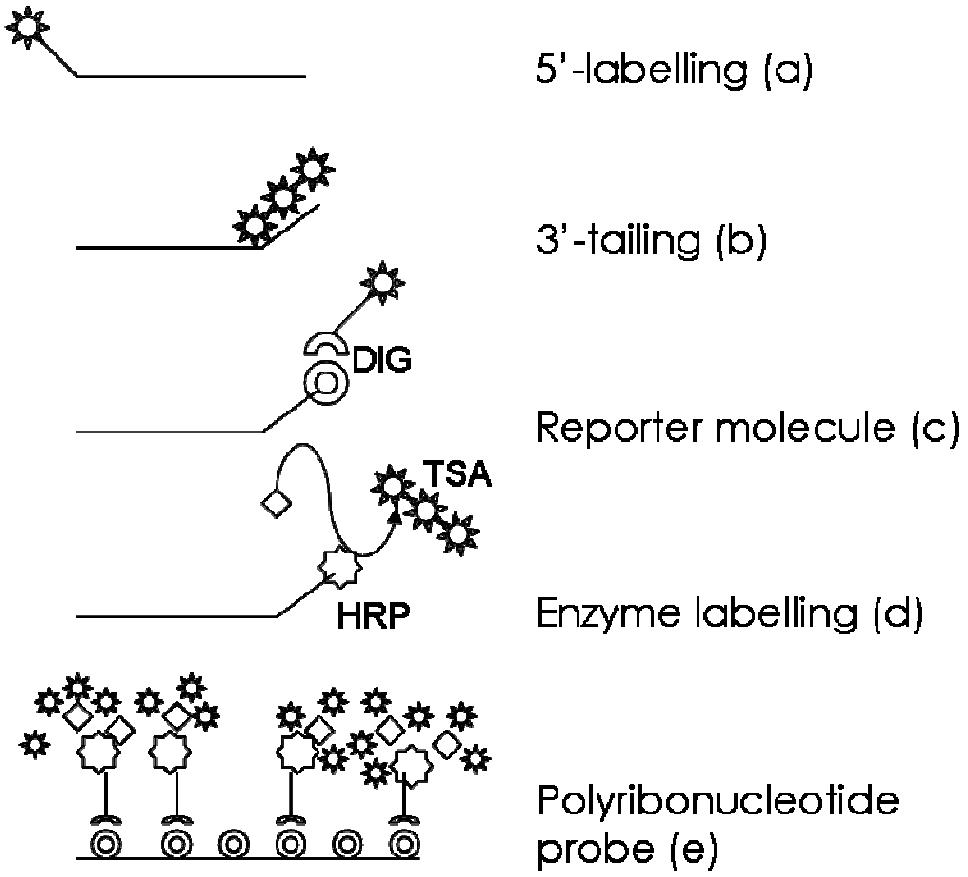

Fig.2 Direct, (a) and (b), and indirect, (c)–(e), labeling of probes using a reporter molecule like digoxygenin (DIG) that is then detected by fluorescent antibody, horseradish peroxidase(HRP) that used fluorescein–tyramide as substrate for the enzymatic signal amplification [Tyramide Signal Amplification (TSA)], or with the combined use of. polyribonucleotide probes, internally labelled with digoxygenin, with the tyramide signal amplification system.

Direct fluorescent labelling is most commonly used and is also the fastest, easiest and cheapest method because it does not require any further detection steps after hybridization (Moter and Gobel 2000). With direct labelling, the probe DNA is synthesized with one or more modified dNTPs (the building block molecules of DNA) that have a fluorochrome chemical side group. By incorporating the fluorochrome directly into the probe, the fluorescent signal can be bound to the target in a single hybridization step (Jain 2004). Given the need to

simultaneously identify several bacterial species, the contemporaneous labelling with different fluorophores could be useful: employing two or three dyes together a colour combination can be obtained which allows to delineate multiple targets by varying the relative contribution of each colour to the total signal (Levsky et al. 2003). It is essential to use fluorochromes with fluorescent spectra that can be clearly differentiated to create a unique spectral signature for each species in images after multi-colour staining. A multi-colour FISH technique was developed for example for the analysis of seven *Bifidobacterium* species in human faeces (Takada et al. 2004).

1.5.1: 5 Probes size and targets

Early probes produced from clones had to be large because they were sparsely labelled in order to allow specific hybridization and because of the methods used for their synthesis and purification, and for this reason, they caused high background fluorescence, problem which could be overcome by pre-treatment with unlabelled nucleic acids to compete for non-specific sites of binding.

An improved signal-to-noise ratio and thus to single-copy detection of genes could also be obtained with a reduction in probe size (Landegent et al. 1987; Lawrence et al. 1988). The utilization of these fluorescent oligonucleotides probes that specifically bind to the ribosomal RNA is nowadays a very useful tool for the detection, identification and enumeration of bacteria.

In the vast majority of applications FISH probes target 16S rRNA. In fact, comparative analysis of 16S (and 23S) rRNA sequences is today the most commonly used method for studying the phylogeny of microrganisms, and such rRNA sequences can be obtained from environmental or medical samples without cultivation. The public

databases now include 16S rRNA sequences, which can be used with specific program packages for probe design (Amann et al. 1998).
The choice of probes for FISH must consider specificity, sensitivity and ease of tissue penetration. A typical oligonucleotide probe is between 15 and 30 base pairs (bp) in length and is generated on an automated synthesizer. Short probes have easier access to their target, but they might carry fewer labels. However, not all bacterial and archaeal cells can be permeabilized by oligonucleotide probes using standard fixation protocols. The accessibility of selected target sites for oligonucleotide probes can be increased by adding unlabelled oligonucleotide probes that bind adjacent to the probe target site. The aim is to unfold the nucleic acid and thus facilitate probe hybridization. These so-called 'helper probes' need to be designed carefully because of their specificity to the respective probe and must have a differentiation temperature (Td) at least as high as the Td of the probe, to prevent dissociation of the helper at stringent hybridization conditions (Fuchs et al. 2000).

1.5.1: 6 Fluorescently labelled oligonucleotide and polynucleotide probes

Fluorescently labelled rRNA-targeted oligonucleotides are used to specifically stain different members of microbial communities. The specificity of the probes ranges from the phylo-type to the kingdom, depending on the targeted region on the rRNA. Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples and the signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. Polinucleotide probes consisting of nearly full-length 16S and/or 23S rRNA genes, each labelled with several fluorochrome molecules, were shown to detect almost all cells present in environmental samples (DeLong et al. 1999). Shorter polynucleotide

probes that target a defined variable region of approximately 250 nucleotides of the 23S rRNA have been shown to allow differentiation among genera (Trebesius et al. 1994). Oligonucleotides probes can be labelled at both 5'- and 3'-ends, and/or several probes applied simultaneously, thereby targeting each rRNA molecule with several fluorophores (Lee et al. 1993).

Poly(ribo)nucleotide probes to discriminate marine bacteria and uncultured marine archea have been successfully applied in the open ocean (Karner et al. 2001). Such probes are RNA transcripts from PCR amplicons of 16S and 23S rRNA genes from either environmental DNA or fosmid clones (DeLong et al. 1999). The use of rRNA-targeted oligonucleotide probes, which are covalently mono-labelled with fluorescent dye molecules, limits the sensitivity of the method and aggravates the use of FISH for identification of prokaryotes with low ribosome content per cell (Wagner et al. 2003). The fluorescently labelled rRNA-targeted polynucleotide probes have been reported to yield higher signal intensities than oligonucleotide probes, and may thus represent a better means of detecting microbes with a low ribosome content (Trebesius et al. 1994). Due to their big dimensions, these probes could lead to a reduction in hybridization efficiency because of both the cell wall and the target site inaccessibility to the longer probes.

1.5.1: 7 FISH with peptide nucleic acid probes

Ribosomal RNA molecules are key functional and structural elements of cells and are highly conserved between closely related species (Perry-O'Keefe et al. 2001). Comparative study of rRNA sequences has allowed evolutionary microbiologists to define the phylogeny and taxonomy of bacteria, yeasts and fungi.

In addition, as rRNAs are present at a high copy number in bacteria, they have often been the target of choice for oligonucleodide probe assays (Amann et al. 1995). Various groups have noted that the degree of fluorescent signal is dependent on the choice of the probe target region within the 16S rRNA molecule, and this variability has been ascribed to poor accessibility of DNA probes to the rRNA target because of the highly stable secondary structures in the rRNA (Amann et al. 1995; Frischer et al. 1996; Fuchs et al. 1998). Furthermore, DNA probes are often limited in their capability to distinguish single nucleotide changes. As a result, it is often difficult to design DNA probes that hybridize efficiently within a given stretch of the rRNA dictated by the nucleotide differences found between closely-related species (Perry-O'Keefe et al. 2001). The use of PNAs has been considered useful in overcoming the variable and sometimes insufficient penetration of probes into bacteria depending on their cell wall characteristics, which is one of the major drawbacks of FISH using oligonucleotide probes, particularly in Gram-positive bacteria (Stender et al. 2001).

Peptide nucleic acid (PNA) molecules are uncharged DNA analogues that bind to nucleic acids much more strongly that oligonucleotides because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar-phosphate backbone of the target molecule (Ray et al. 2000).

The unique character of PNA allows these probes to hybridise to target nucleic acid molecules more rapidly and with higher affinity and specificity compared to DNA probes (Jain 2004). This highly sensitive approach detects rRNA in bacterial cells long after cell death (Moter et al. 2000). Moreover, PNA probes hybridize virtually independently of the salt concentration, being ideal for targeting nucleic acids with high degree of secondary structure, like rRNA, since hybridization can be

performed efficiently in a low salt buffer. Under these conditions, the stability of the secondary structures within the target rRNA is decreased, thereby allowing PNA probes to hybridize to less accessible targets. A standardized PNA FISH procedure also increases the possibility of simultaneous identification of different organisms (multiplex identification) using PNA probes labelled with different fluorophores (Perry-O'Keefe et al. 2001). At this time high prices and specificity problems are slowing the application of PNAs to FISH (Amann et al. 2001).

1.5.1: 8 Probes accessibility to rRNA

One of the main problems of FISH, besides low cellular ribosome content and impermeability of cells walls, is the inaccessibility of probe target sites (Fuchs et al. 1998). Initially they were supposed to be hindered by rRNA-rRNA interactions, as well as by interactions of the rRNAs with ribosomal proteins, due to the densely packed three-dimensional structure of the ribosome (Beherens et al. 2003; Wimberly et al. 2000). Later, Inacio et al. (2003), after studying the effects of different hybridization and cell storage conditions, observed that, being FISH performed in a strongly denaturing environment, the 3D structure of the native small ribosomal subunit is not relevant to probe accessibility, and that, for the same reason, the influence of protein-RNA interactions on target site accessibility can be neglected. It was noticed that intrahelix, secondary base interactions are more important than tertiary rRNA-rRNA contacts (Inacio et al. 2003). Frequently, probes were intended to be as specific as possible, targeting particular species or genera, or even just particular 16S rDNA sequences that were retrieved from the environment. Those probes necessary target the most variable regions of the 16S rRNA molecule which often are even the most inaccessible sites (Fuchs et al.

1998). Fuchs et al. (1998) found five regions in *Escherichia coli* where accessibility seemed to be very high: position 38 to 108 (except for the terminal loop region of helix 6), position 181 to 215, position 316 to 359, position 871 to 933, and positions 1383 to 1427 and 1473 to 1517. They found that the loop region of helix 6, the loop region and the distal 3' side of helix 18 and the 5' side of helix 23, which are among the most important target sites being the evolution less conserved, are not very accessible. In this study, all probes were arbitrarily grouped according to their relative fluorescences into six classes of brightness, where the fluorescence of the probes was expressed as a percentage of the brightest probe (Eco1482). Most probes in the first two classes are directed against five regions where accessibility for oligonucleotide probes seems to be very high, so it is possible to consider the brightest probes as the most accessible ones. In this contest, probe Eub338, which is the most widely employed probe for FISH detection of *Bacteria*, has been set in the III class, with a 58% of Eco1482 brightness. The fluorescent signals reported by Fuchs et al. (1998) should mainly reflect differences in 16S rRNA accessibility. Changes in accessibility are often steady along the primary structure of the 16S rRNA, but can also be rapid and are therefore quite unpredictable. Moreover, they noticed that the almost inaccessible target sites are often in the periphery of the secondary structure model, including many loops, whereas regions in the centre of this model seem to be more readily accessible. Further studies investigated the possibility of using the 23S rRNA as a probe target molecule because, despite its length of approximately 1,500 nucleotides, it is sometimes impossible to find suitable signature sites on the 16S rRNA for the identification of the organisms of interest (Fuchs et al. 2000b). The 23S rRNA, with its length of approximately 3000 nucleotides, would be the ideal alternative as a probe target

molecule (Fuchs et al. 2000b). The authors demonstrated that in *E. coli* the 23S rRNA is more accessible to oligonucleotide probes than the 16S rRNA in the small subunit of the ribosome and, for this reason, a 23S rRNA-targeted probe is valuable in increasing the significance of *in situ* identification.

1.5.1: 9 Transferability of E. coli accessibility data to other organisms

Due to the high evolutionary conservation of the rRNA, these findings on the 23S rRNA and 16S rRNA *in situ* accessibility for *E. coli* can be extrapolated to other microrganisms. The transferability of *E. coli* accessibility data to other organisms has been studied for organisms of all three domains of life: *Bacteria, Archea* and *Eucarya* (Beherens et al. 2002), and the correlations analysis clearly showed that the *in situ* accessibility maps are more similar for philogenetically more-related organisms. For all three prokaryotes examined by Beherens et al. (2002) regions of the 16S rRNA with high accessibility are positions 285 to 338 (helices 13 and 14), positions 871 to 925 (except helix 30 target positions), and positions 1248 to 1283. Seven smaller regions of good accessibility are located on helices 2, 3, 7, 9, 20, 23, 26, 27 and 31. As shown by Inacio et al. (2003), the accessibility to 26 rRNA in yeasts (*S. Cerevisiae*) follows the same general trend observed for 16S rRNA of *E. coli*: the most conserved stretches of the region studied are more accessible, and the most variable areas often show medium to low accessibility. Otherwise, the *in situ* accessibility does not depend exclusively on a probe target site location inside or outside the ribosome, as suggested by Beherens et al. (2003). For example, fixation and hybridization significantly increase the accessibility of 16S rRNA target sites to probe, causing massive conformational changes within the ribosome (Beherens et al. 2003). Inaccessible target sites could

even be likely opened up by conformational changes introduced by hybridization of helper probes (Fuchs et al. 2000).

There is even a link between the type of label and accessibility. Studies performed by Beherns et al. (2002) and by Fuchs et al. (1998) demonstrated that Cy3-labelled oligonucleotides have higher *in situ* accessibility in *E. coli*, in comparison to the triphenilmethane derivative carboxy-fluorescein, due to the more linear structure, which could reduce steric hindrance and thereby facilitate probe binding to the target.

1.5.1: 10 Hybridization efficiencies of FISH probes

Although the accessibility maps from the studies cited have been used for the selection of probe target sites in FISH experiments, recent studies have demonstrated that it is not worth to eliminate rRNA target regions *a priori*, even if they were previously reported as seemingly inaccessible, because these sites could be strongly influenced by the kinetics of the hybridization reaction (Ylmaz and Noguera 2004; Ylmaz et al. 2005). In fact, the hybridization efficiencies of FISH probes can be defined as a function of not only the accessibility of the target site, but also the thermodynamic affinity of the probe to the target site, defined by the predictable free energy change of the overall reaction ($\Delta G°_{overall}$), which describes the stability of the DNA/RNA hybrid and can be correlated with the brightness of a hybridized probe. $\Delta G°_{overall}$ is obtained from the free energy change for the individual reactions ($\Delta G°_i$): the binding of the probe to the complementary site available (reaction 1), the reversible unfolding of the target region (reaction 3) and the folding-unfolding mechanism for the DNA probe (reaction 2). $\Delta G°_i$ can be calculated as $\Delta G°_i = -RTlnK_i$, where R is the ideal gas law constant, T is the hybridization temperature, and K_i is the equilibrium constant for each reaction. A more negative $\Delta G°_{overall}$ represents a

greater concentration of the hybrid and, thus, greater brightness from the hybridized probes (Ylmaz and Noguera 2004).

For this reason, $\Delta G°_{overall}$ is a strong predictor of hybridization efficiency and it has been noticed that an affinity above the theoretical threshold of -13.0 Kcal/mol for maximum hybridization efficiency should be targeted for rational design of FISH probes to maximize the possibility of satisfactory sensitivity (Ylmaz et al. 2005). Furthermore, kinetics limitations imposed by structural restraints in the ribosome could be overcome by extending the incubation period from 3h to 96h, or using formamide, so that all regions of the 16S rRNA of *E. coli* can be made accessible, thanks to the surmounting of thermodynamic and kinetic barriers (Ylmaz et al. 2005).

1.5.1: 11 Use of FISH in food samples

There is an increasing need for rapid detection and enumeration of marker organisms that run to completion within a single working day in the food and drinking water industries (Ootsubo et al. 2003). A major disadvantage of culture methods is the time needed to produce results. Generally, they require incubation of culture plates for several days. Another significant disadvantage of these methods is the failure to detect viable but non-culturable organisms. Therefore, it raises doubts that culture-based methods recover sub-lethally injured cells that may occur in heat-treated products such as pasteurized milk. In order to monitor on-line sanitation practices, the industry needs rapid and reliable methods to ascertain the microbiological quality of foods and ingredients. Fluorescent oligonucleotide probe hybridization is becoming an important tool also in food microbiology to identify specific microrganisms in mixed communities without the need for isolation in pure cultures. The use of FISH allowed, for example, a rapid and accurate enumeration of *Pseudomonas,* facilitating the

identification of specific contamination sources in dairy plants, the accurate validation of pasteurization treatment and the prediction of shelf life of pasteurized milk (Gunasekera et al. 2003).

Moreover, the use of group- and species-specific oligonucleotide probes for *in situ* hybridization has provided an insight to the microbial composition of Gruyere cheese surface (Kolloffel et al. 1999). Although the FISH analysis was performed on a cheese suspension and not directly in the food matrix , the study allowed the detection of most bacteria with bacterial probe Eub338, even in mature cheese from which only a smaller proportion of the bacteria were culturable (Kolloffel et al. 1999).

This technique was recently applied for a screening of the occurrence of *Lb. plantarum* on olives to be used in natural fermentations. However, due to the high detection limit of the method, the species could not be detected even though a large number of samples were screened (Ercolini et al. in press). Moreover, technical problems arose in this study for the optimization of cell permeabilization condition due to the simultaneous occurrence of Gram positive and Gram negative bacteria (Ercolini et al. in press).

FISH is useful in wine production too, where the early knowledge of the microbiological conditions allows the application of corrective measures before the spoiling becomes irreparable. Therefore, rapid and accurate identification of LAB permits a better monitoring of the fermentation process, preventing the risk of alteration and the probability of the occurrence of malolactic fermentation, leading therefore to a better quality of the final wine (Blasco et al. 2003).

The use of FISH and, in particular, the application of fluorescently labelled PNA oligomers, has also been reported to be a powerful method for identifying colonies of *Brettanomyces,* a well recognized

wine spoilage yeast that causes an undesirable flavour (Stender et al. 2001).

Despite the huge background of knowledge and application, the use of FISH in food microbiology has been restricted to the identification of bacteria isolated from food or in food suspension, therefore losing spatial data which might provide important information on flora development in food ecosystems.

Ercolini et al. (2003a) developed a 16S rRNA fluorescence *in situ* hybridization (FISH) method for cheese, to allow the detection *in situ* of microrganisms within the dairy matrix. An embedding procedure using a plastic resin was applied to Stilton cheese, providing intact embedded cheese sections withstanding the hybridization reaction. The technique has the potential to study the spatial distribution of microbial population *in situ* in foods, especially where the matrix is too fragile to allow manipulation of cryosections. 16S rRNA sequences could be used for species-specific probe design for the location of specific groups of bacteria within the matrix, and for the investigation of relationships within specific groups of bacteria.

The authors developed probes for the specific detection of some species of lactobacilli, lactococci and leuconostocs and demonstrated that in Stilton cheese the spatial distribution of specific colonies is not homogeneous but depends on the specific site. The availability of such information for dairy products after FISH investigation may play a key role for the detection of the sites of actual growth of certain species and can be of potential help in detecting zones of appropriate acidification, aroma development and production of antagonistic substances in food products. Moreover, the monitoring and analysis of spatial distribution of contaminant species might also be achieved by this approach, which represents a very important step forward in studying the development of microbial populations in food with the

ultimate aim of process optimization and quality assurance of the final product (Ercolini et al. 2003, a-b).

1.5.1: 12 Applicability of FISH to biofilms

Although quantitative FISH has provided new insights into the structure and dynamics of microbial communities, it suffers from tediousness and limited accuracy for samples containing densely aggregated cells like biofilms (Daims H. et al. 2001). Biofilms are formed after rapid attachment and growth of microrganisms on a broad range of surfaces in contact with natural fluids. Biofilms architecture and the physiological status of the cells contained within these structures are of prime interest for clinical, industrial, and environmental microbiology. For analyses of the spatial arrangement of bacteria in a multi-species biofilm, techniques which use specific bacterial cell markers and maintain the biofilm's natural architecture are required (Thurnheer et al, 2004).

Quantitative data on the abundance of *in situ* stained microrganisms in complex samples are mostly obtained by time consuming manual microscopic counting. The accuracy of this quantification approach is relatively low in densely colonized biofilms, which probably represent the most common life form of microrganisms on Earth (Wagner et al. 2003). This problem can in part be ameliorated by using confocal laser scanning microscopy (CLSM) for the detection of probe-labelled cells. However, even if optical CLSM sections are recorded, it is not feasible to manually count a sufficient number of cells in each hybridization experiment in a reasonable time period to obtain statistically reliable results. Even semi-automated digital image analysis tools are unable to efficiently count cells in dense clusters or biofilms because single-cell recognition within these structures cannot be automated. This problem can be circumvented by measuring the areas of specifically

stained bacteria in randomly acquired optical CLSM sections. The abundance of a particular population is then expressed as a fraction of the area occupied by all bacteria (Daims et al. 2001).

The utilization of genus-specific, fluorescently-labelled oligonucleotide probes can be a very useful tool for the study of particular kinds of biofilms, such as those formed in the gastrointestinal human tract. FISH has been applied with this aim for the hybridization of the bacterial rRNA extracted from a faecal sample or fermentation fluids (Langendiik et al. 1995), and has also been used to determine changes in populations of bifidobacteria, lactobacilli, clostridia, bacteroides, streptococci and *Escherichia coli* in human faecal extracts (Rycroft et al. 2001). Moreover, combining FISH with confocal laser scanning microscopy (CLSM) , the development of a simultaneous analysis of the spatial distribution of both Gram-negative and Gram-positive bacteria in biofilms has been possible (Thurnheer et al 2004).

1.5.1: 13 Conclusions and future perspectives

As a consequence of the continuous improvements, FISH is nowadays a very useful tool for studies in microbiology. Through the use of species-specific probes, it is possible to identify different microrganisms in complex microbial communities, giving a solid support to the understanding of interspecies interaction. The widespread use of FISH as an application directed to gaining information on the spatial distribution of microbial populations will allow to study the contribution of each species to the organization and functioning of the multispecies bacterial communities. This is important from different points of view and in different fields of application. The knowledge of the composition and distribution of microrganisms in natural habitats can be interesting for ecological reasons in microbial ecology, and for safety and technological aspects

in food microbiology. This technique will be a relatively rapid method to evaluate the presence and activity of pathogens, or other undesirable microrganisms, directly in the sample studied (food, water, wastes, soil), but it will also be a valid approach to study the activity of bacteria involved in technological processes (e.g. food fermentations) or important for human health (e.g. *Bifidobacteria*).

FISH is the very *in situ* technique, and will keep the potential to zoom in on the composition of complex microbial associations in natural ecosystems.

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2 Aim of the thesis

The relation between microorganisms and food excited much interest since the existence of bacteria has been observed. So far, many efforts have been put in trying to investigate this association, especially through traditional, culture-based methods. However, further the knowledge of structure and dynamics of the whole microbial community, would promote better understanding of how food ecosystems are influenced by microbial growth and metabolism. For instance, greater control over microflora composition would make it possible to better select for specific organoleptic properties, to prevent quality defects or spoilage and to guarantee food safety. Trying to shed light on what traditional culture-dependent methods left hanging, aim of this PhD thesis, was to find new ways to study microorganisms in food. As they are fast and potentially more exhaustive, culture-independent methods are well suited for analysing microbial communities over time and may provide the possibility of exploring food microflora in detail. The actual applicability of those methods to food ecosystems was challenging evaluated in different fermented food. Fluorescent staining, FISH (fluorescence in situ hybridization) and LH-PCR (length heterogeneity PCR) have been used to gain a deeper knowledge of microbial composition and dynamics of different food matrices: two different fermented foods such as artisanal beer and Parmigiano Reggiano, and a complex dairy ecosystem such as natural whey starter for Parmigiano Reggiano.

3. RESULTS

3.1 FISH in food microbiology

Benedetta Bottari, Danilo Ercolini, Monica Gatti, and Erasmo Neviani
T. Liehr (ed.) Fluorescence In Situ Hybridization (FISH) – Application Guide.

3.1.1 Abstract

As a consequence of the continuous improvements, FISH is nowadays a very useful tool for studies in microbiology. The knowledge of composition and distribution of microorganisms in natural habitats has a key role for safety and technological aspects in food microbiology. FISH can be used here directly in the sample studied as a relatively rapid culture-independent method to evaluate the presence and activity of pathogens, or other undesirable microorganisms. Moreover, molecular cytogenetics is a valid approach to study the activity of bacteria involved in technological processes (e.g. food fermentations) or important for promoting human health. In this chapter we present an overview on FISH protocols for the microbiological analysis of food.

3.1.2 Introduction

Foods are teeming with microorganism that may be innocuous, pathogenic threats, spoilage agents, or beneficial microorganisms driving fermentations or acting as biocontrol agents. Historically, the overarching priorities in this field have been the destruction and control of undesirable organisms in foods or promotion of the growth and activity of desirable microbes. More recently, emphasis has been placed into the understanding of the interactions of microbes within their human/animal hosts and in highlighting the impact of food, processing conditions, and storage environments on bacterial responses that promote activity, survival or destruction (Klaenhammer 2006).

In the last few years, several studies have been carried out in order to find new ways of approaching to food microbiology (Cocolin and Ercolini 2008). Great efforts were made in order to evaluate the presence and the activity of both pathogens and bacteria that are involved in technological processes (e.g. food fermentations) or important for promoting human health.

The diversity of microbial populations studied by traditional methods, like plating on selective media, commonly detect the most frequently occurring organisms that can grow to a detectable level by forming colonies on selective media. However, such methods are not able to recover the less abundant components of the micro flora (Steele et al. 2006). It is widely accepted that plate culturing techniques reveal a small portion, i.e., that viable and cultivable of the true/real microbial population in natural ecosystems. This could essentially be explained by two ecological factors: (i) the inability to detect novel microorganisms, which might not be cultivable with known media and (ii) the inability to recover known microorganisms which are either stressed or actively growing but which enter an non-cultivable state (Giraffa 2004). Furthermore, the assessment of the microbial composition of complex microbial communities through traditional methods is laborious, time-consuming, and prone to statistical and methodological errors (Moter and Gobel 2000). In fact, according to classical microbiological procedures it is necessary to cultivate a microorganism from the original matrix on the appropriated medium and to isolate it from the plates corresponding to the so-called countable dilutions. Thus, traditional commercial growth media, could be too generic and not always enough selective to differentiate species or biotypes present at different concentrations. For such reasons, a certain extent of the microbial population present in a food matrix may be underestimated. Nonetheless, such information is often crucial in

the understanding of the microbial evolution occurring in a fermented food, e.g. the identification of the microbial components taking place during different phases of ripening.

Moreover, environmental modification occurring during cultivation on synthetic media could affect the structure of microbial communities and thus limit the complete view of the considered ecosystem (Bottari et al. 2006). Similarly, understanding the ecology of complex microbial communities, such as those present in foods, requires even studies on the activity and distribution of microbes; these should be performed directly in minimally disturbed samples (Amann et al. 1998).

Recently, there is an increasing need of rapid and reliable methods to assay the microbiological quality of foods, compatibly with the demands of producers (Cocolin and Ercolini, 2008). In this context, several methods avoiding cultivation have been developed, and fluorescence in situ hybridization (FISH), which has seen some major improvements since its birth, is nowadays one of the most used techniques in such microbiological studies. After the first medical and developmental biology origin, FISH was applied primarily in environmental bacteriology (Amann et al. 1995) and to a lesser extent in protist ecology (Lim et al. 1996). Nowadays, thanks to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental microbiology (Amman et al. 2001; Pernthaler et al. 2001). In particular, FISH has so far a large number of applications that include the investigation of microbial symbiosis, the analysis of microbial diversity in environmental samples, the evaluation of the presence of bacteria in wastewater treatment plants (Amann et al. 2001), the identification of bacteria relevant in diagnostic medicine, and the detection of pathogens within human and animal tissues (Moter and Gobel 2000). The selection of particular regions of the rRNA molecule enables then

phylogenetic specificity to be varied from the domain to the subspecies level (DeLong et al. 1989; Amann et al. 1990), even if, because of the relatively slow mutation rate of rRNA, this molecule generally possesses no target sites that differentiate between strains of prokaryotic species (Wagner et al. 2003). Due to these characteristics, fluorescent oligonucleotide probe FISH has become more and more used also in food microbiology to trace specific microorganisms in mixed communities without the need of any preliminary bacterial cultivation procedure. With respect to food microbiology the use of FISH has provided an insight into the microbial community composition of different, non fermented and fermented foods such as cheese (Kolloffel et al. 1999) olive (Ercolini et al. 2006) and wine (Blasco et al. 2003, Stender et al. 2001). FISH allowed the detection of most bacteria even in samples where cultivable bacteria were at a low level among the total microbial population, and in samples where the early knowledge of the microbiological conditions allowed the application of corrective measures before the spoiling becomes irreparable.

FISH technique allows also a quick and specific detection of food-spoilers in the production of beer and fruit juice (Thelen et al. 2002, 2003). This technique together with the use of oligonucleotide probes is highly useful for the in situ detection of food pathogens (Schmid et al. 2005, Stephan et al. 2003). Notably, FISH has been applied in the enumeration and identification of specific contamination sources in factory processes, e.g. factory plants (Gunasekera et al. 2003). However, despite the huge background of knowledge and application, the use of FISH in food microbiology has been restricted to the identification of bacteria isolated from food or food suspension, and therefore missing information on flora development in food ecosystems (e.g. data regarding the distribution of bacterial cells along the food

product). Recently, Ercolini et al. (2003a) described a novel FISH method offering the potential to study the spatial distribution of microbial population in situ in food matrix that is based on an embedding procedure of cheese samples involving a plastic resin. These authors developed species-specific probes based on 16S rRNA sequences, which allowed the location of specific groups of bacteria within the food matrix, and the investigation of relationships within specific groups of bacteria, proving the dependence of microbial spatial distribution on specific site. This kind of FISH investigation, which is described in the following, may lead to an improvement of process optimization and quality assurance of the food product, enabling the detection of sites of actual growth of contaminant species and species of technological interest (Ercolini et al. 2003, a-b).

3.1.3 Outline of the procedure

FISH with rRNA target probes for the in situ analysis of single microbial cells has been developed as a culture independent "non-PCR-based" molecular technique for the simultaneous visualization, identification, enumeration, and localization of individual microorganisms from all fields of microbiology (Amann et al. 1990, 2001; Moter and Gobel 2000). A typical FISH procedure includes four main steps enclosing: i) fixation and permeabilization of the sample; ii) hybridization; iii) washing steps directed to remove unbounded probe; iv) detection of labeled cells by microscopy or flow cytometry (Amann et al. 2001).

Fixation and permeabilization of bacterial cells are preceding the hybridization phase, allowing penetration of the fluorescent probes into the cell as well as protecting the RNA molecules from degradation by endogenous RNAsi (Moter and Gobel 2000). The fixing agent can be used directly to cover the sample settled on a membrane filter

(Glockner et al. 1999), or mixed with the sample before incubation, sedimentation by centrifugation, re-suspension, spotting on the glass slides followed by air dehydratation (Amann et al. 1990). Sometimes, an enzymatic treatment could be necessary, e.g. in Gram positive bacteria (Schonhuber et al.1997; Wagner et al. 1998, Krimmer et al. 1999). Furthermore in order to avoid cell loss or insufficient adhesion of specimens to glass slides, the glass surfaces are often treated with coating agents such as gelatin (Amann et al. 1990), poly-L-lysine (Lee et al. 1999) or silanating agents (Moter et al. 1998). The hybridization takes place in a dark humid chamber, usually at temperatures between 37°C and 50°C for times ranging from 30 min to several hours. Slides are subsequently briefly rinsed with distilled water in order to remove unbounded probe, mounted in anti-fading agents to prevent fluorescence "bleaching" (Moter and Gobel 2000), visualized and documented.

3.1.4 Materials

A wide variety of probes is currently being used to examine natural bacterial communities such as those of food matrix. DNA probes require stringent hybridization conditions, specific to each individual probe, needing the optimization of hybridization protocols case by case. As an example, chemicals and solutions reported here are referred to the universal probe Eub338 (Amann et al. 1990). Apart from standard equipment the following more specialized reagents are needed (listed in alphabetical order).

Chemicals

The probes which can be used for FISH in different bacteria are listed in Table 1.

- EDTA (ethylene-diamino-tetra-acetic acid)
- Ethanol
- Formamide (Methanamide)
- Lysozime
- NaCl (sodium chloride)
- Na Citrate (sodium citrate)
- Paraformaldehyde
- PBS (Phosphate buffered saline: 8 g NaCl, 0.2 g KCl, 1.44 g, Na_2HPO4, 0.24 g KH_2PO_4 in 800 ml of distilled H_2O)
- Proteinase K
- SDS (sodium dodecyl sulfate)
- TE buffer (10 mM Tris, bring to pH 7.5 with HCl, 1 mM EDTA)
- Tris-HCl (Tris Hydrochloride)

Solutions to be prepared

- Hybridization buffer:
  - 0.9 M NaCl
  - 0.01% SDS
  - 20 mM Tris-HCl pH 7.2
  - formamide at the opportune concentration
- Lysozyme solution: dissolve 1 ml of lysozyme 69490 U in 1 ml of 5 mmol l^{-1} EDTA, 100 mmol l^{-1} Tris-HCl, pH 7.5)
- Proteinase K solution: dissolve 10 mg in 1 ml of re-distilled sterile water
- Washing buffer:
  - 20 mM Tris-HCl pH 7.2
  - 0.01% SDS
  - 40 mM NaCl
  - 5 mM EDTA

Probe	Specificity	Sequence (5′–3′) of probe	Target site (rRNA positions)	% FA	References
EUB338	Most bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)a	0–60	Amann et al. 1990
BIF164	*Bifidobacterium*	CATCCGGCATTACCACCC	16S (164–181)a	0	Langendijk et al. 1995
BRE1239	*Brevibacterium*	TCTCTCTGTACCAGCCAT	16S (1239-1257) a	30	Kolloffel et al. 1997
Bet42a	Betaproteobacteria	GCCTTCCCACTTCGTTT	23S (1027–1043)a	35	Manz et al. 1992
Gam42a	Gammaproteobacteria	GCCTTCCCACATCGTTT	23S (1027–1043)a	35	Manz et al. 1992
HGC69a	Actinobacteria	TATAGTTACCACCGCCGT	23S (1901–1918)a	25	Roller et al. 1994
CAMP653	*Campylobacter*	CTGCCTCTCCCTYACTCT	16S (653–670)	35	Schmid et al. 2005
LGC354ab	Firmicutes	YGGAAGATTCCCTACTGC	16S (354–371)a	35	Meier et al. 1999
LactV5	*Lactoccoccus lactis*	GCTCCCTACATCTAGCAC	16S (821-839) a	25	Ercolini et al. 2003b
LU2	*Leuconostoc*	GATCCATCTCTAGGTGACGCCG	16S (221-242) a	0	Niessen et al. 1994
Lpara	*Lactobacillus casei, L. paracasei*	GTTCCATGTTGAATCTCGG	16S (94-113)a	0	Blasco et al. 2003
Lbrev	*Lactobacillus brevis*	CATTCAACGGAAGCTCGTTC	16S (64-83) a	0	Blasco et al. 2003

LbpV3	*Lactobacillus plantarum*	CCGTCAATACCTGAACAG	16S (468-486) a	25	Ercolini et al. 2003b
Lis-637	*Listeria*	CACTCCAGTCTTCCAGTTTCC	16S	35	Schmid et al. 2003
Lis-1255	*Listeria, Brochothrix*	ACCTCGCGGCTTCGCGAC	16S (1255-1272) [a]	35	Wagner et al. 1998
OENOS 5/1	*Oenococcus oeni*	GACCTCATCGGAATTAAC	5S	0	Hirschhauser et al. 2005
OENOS 5/2	*Oenococcus oeni*	TACTTTGGGCCCTGACA	5S	0	Hirschhauser et al. 2005
OENOS 5/3	*Oenococcus oeni*	ACCTTGCAACAGGCGTT	5S	0	Hirschhauser et al. 2005
ENT	*Enterobacteriaceae*	TGCTCTCGCGAGGTCGCTTCTCTT	16S (1251–1274)[a]	20	Ootsubo et al. 2002
Eco1482	*Escherichia coli*	TACGACTTCACCCCAGTC	16S (1482-1499) [a]	30	Fuchs et al.
Pae	*Pseudomonas* spec.	TCTGGAAAGTTCTCAGCA	16S (997–1014)[a]	0	Amann et al. 1996
PS	*Pseudomonas* spec.	GATCCGGACTACGATCGGTTT	16S (1284–1304)[a]	0	Gunasekera et al. 2003
Sth	*Streptococcus thermophilus*	CATGCCTTCGCTTACGCT	16S (69–87)[a]	25	Beimfohr et al. 1993
EUK	*Eukarya*	ACCAGACTTGCCCTCC	18S (502–517)[a]	20	Amann et al. 1990

Table 1: Some of the oligonucleotide probes used for FISH analysis in food samples.

[a] *Escherichia coli* rRNA numbering (Brosius et al. 1981).

[b] *Bacillus subtilis* rRNA numbering (Cannone et al. 2002)

[c] Formamide concentration (FA) in the *in situ* hybridization buffer (vol/vol).

3.1.5 Protocol

Hybridization and washing requires precise temperature control to prevent non specific hybridization at low temperatures and loss of correctly hybridized probes at high temperatures. The optimal hybridization and washing temperature must be chosen depending on the melting point of the selected probe and on the target site accessibility. In fact, temperature does not only affect the dissociation of a probe, but also affects the conformation of the targeted rRNA or DNA and thus, the accessibility of the targeted rRNA to oligonucleotide probes (Fuchs et al 1998; Tang 2005). In general, the higher the temperature the more stringent hybridization and washing temperatures have to be. Temperature and time of hybridization and washing presented here, are an example and must be optimized case by case.

3.1.6 Preparation of liquid samples or suspension of solid samples for FISH analysis

Fresh liquid samples

This could be for example whey starter or milk, and it should be prepared as follows:

1. Centrifuge an aliquot (i.e. 0.3-0.5 ml) of the sample (7000 g for 5 min)
2. Discard supernatant, re-suspend and wash cell pellets in an equal volume of TE buffer. Repeat step 1.
3. Discard supernatant and re-suspend the pellet in an equal volume of 1x PBS.
4. Add para-formaldehyde (4%, -20°C) 1:3 and fix for 1h at +4°C; repeat step 1.

5. Wash pellet with 1 ml of PBS 1x. Repeat step 1.
6. Discard supernatant and re-suspension pellet in 50% (v/v) ethanol/PBS
7. Store at -20°C until further FISH analysis.

3.1.2 Suspension of solid samples

E.g. cheese or meat has to be prepared as follows:

1. Disperse 5 -10 g of each sample in 1:10 Na Citrate solution pH 7.2 by stomaching for 2 min.
2. Centrifuge 1 ml of stomached mixture at 7000 g for 5 min.
3. Discard supernatant and re-suspend the pellet in an equal volume an equal volume of TE buffer. Repeat step 2.
4. Wash pellet with 1 ml of PBS 1x. Repeat step 2.
5. Add para-formaldehyde (4%, -20°C) 1:3 and fix for 12h at +4°C.
6. Centrifuge at 10.000 g for 5 min.
7. Discard supernatant and re-suspend pellet in 50% (v/v) ethanol/PBS
8. Store at -20°C until further FISH analysis.

Preparation of solid samples for FISH analysis

Solid samples can be prepared in different ways depending on the nature of the matrix. Basically, the solid samples must be sliced in very thin sections (5-30 μm thick) to be analyzed by FISH. The sections should:

- endure the hybridization conditions (solutions, temperature, detergents etc.);
- assure the integrity of the matrix;
- allow probe entry with good yield of hybridization
- not to alter the microbial colony distribution within the sample.

Therefore, different sectioning procedures can be taken into account. For resistant matrices a simple cryo-sectioning procedure can be

enough. Alternatively, embedding agents can be used such as paraffin. The latter do not always work for delicate but microbiologically complex samples such as solid foods. However, FISH can be applied to tissues or foods embedded in a cold polymerizing resin. The sections have a long shelf life and it is often possible to avoid enzymatic pre-treatment of the samples. Some protocols have been developed for tissues (Moter et al. 1998) and cheese (Ercolini. 2003a-b) using an embedding procedure with a cold polymerising glycol methylacrylate (GMA) resin (Technovit 8100, Kuzler, Wehereim, Germany) according to the manufacturer's instructions.

FISH on liquid samples or suspension of solid samples

1. Spot about 20 µl of fixed cell suspension on poly-L-lysine coated slides.
2. Dry in oven at 46°C for 10 min.
3. Dehydrate in an ethanol series by covering the spots with about 50 µl of 50, 80 and 100% ethanol solutions for 3 min each and air dry
4. Enzymatic treatment: specimens can be treated by covering the spots either with 10 µl of proteinase K (10 mg ml $^{-1}$) for 10 min at 37°C, or with 30 µl of lysozyme (1 mg ml $^{-1}$) for 5 min at room temperature.
5. Stop of reaction with a washing with ice cold PBS before drying.
6. Add 10 µl of the hybridization buffer containing 10 ng of selected rRNA probe onto the dry specimen.
7. Incubate slides in a dark humid chamber at 45°C overnight.
8. Remove of unbound oligonucleotides by incubating the slides in pre-warmed washing buffer at 45°C for 15 min
9. Rinse the slides by pipetting about 500-1000 µl of sterile water on the surface.
10. Air dry.
11. Embed the samples in mounting oil.

12. Evaluate the slides with an epifluorescence microscope equipped with a 100x objective and opportune filter sets.

3.1.7 Results

Aiming to evaluate microbial population in food, we have to distinguish at least two kinds of food: liquid and solid. Moreover we have to consider fermented food, with or without microbial starter, where beneficial microorganisms drive fermentations or act as biocontrol agents, and non-fermented food where microorganism may be innocuous, pathogenic threats or spoilage agents.

FISH on liquid food samples or on suspension of food solid samples is quite simple and could be used for example to follow population dynamics in complex ecosystems. After an efficient sample preparation an in situ analysis of complex sample materials can be performed on morphologically intact cells. This is an appropriated approach when studying the dominant microbial populations such as those found in fermented foods, where the overcoming of specific species on other microbes, naturally present, should be studied to better know the fermentation processes (Cocolin and Ercolini 2008; Cocolin et al. 2007).

In particular, FISH may be rather easily and effectively used to analyze liquid starter culture. Starter cultures are of great industrial significance since they play a crucial role in the manufacturing of fermented food and in the development of their flavour and texture. FISH technique could be a useful approach in order to study selected starter cultures and subsequently evaluate their effectiveness and/or performance during production processes. Although, FISH procedures might be even more effective in investigating the microbial composition of natural starter cultures, which are highly variable with respect bacterial composition and less well-known.

One useful application of this technique is the possibility to study the composition of natural whey starter traditionally used to produce PDO (Protected Designation of Origin) Italian cheese such as Parmigiano Reggiano and Grana Padano. For example, with the simultaneous use of two species-specific probes (Tailliez and Tailliez, personal communication), labeled with different dyes, it has been possible to highlight in natural whey starter the abundance of *Lactobacillus helveticus* respect to *Streptococcus thermophilus* (Figs. 1 a and b). These results are convincing and can be easily interpreted because the number of microbial cells typically present in this dairy starter is about 10^8-10^9 cell/ml. Otherwise, FISH has a significant limit of detection when coupled with fluorescence microscopy (10^6 cell/ml), which makes this technique unsuitable to monitor bacterial cells present in very low amount (e.g., pathogens). For example, few hybridized cells per field have been detected in an experimental cheese sample artificially inoculated with 10^6 cells/ml of *E. coli* and analyzed by FISH with *E. coli* specific probe (Fig 2). FISH-based testing kits containing probes specific for pathogens (Stephan et al. 2003) are commercially available, but require pre-enrichment of sample in nutrient substrate. Thus, this FISH-based kits should not be considered anymore as a "culture-independent" procedure. The detection limit can be overcome when the very in situ hybridization is done. In fact, a sample embedding procedure withstanding the hybridization reactions enables to detect the microorganisms in situ within the food matrix (Fig. 3). In this way, the technique has the potential to study the spatial distribution of microbial population in situ in foods allowing for the location of specific groups of bacteria within the food matrix, and the investigation of relationships existing within specific groups of bacteria.

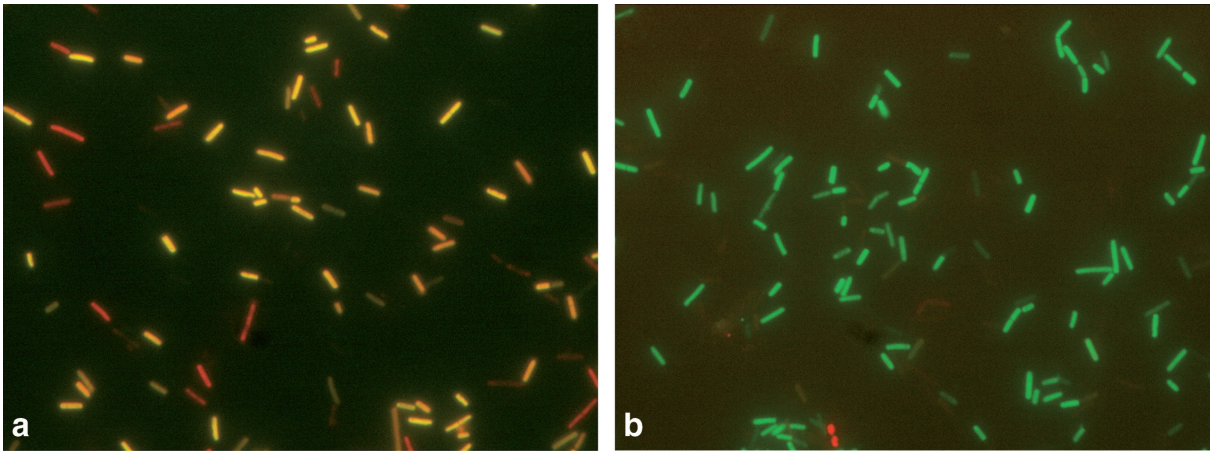

Fig. 1 FISH of a natural whey starter. a Simultaneous use of the probes Eub338 (red label) and Lbh1 (green label). *Lactobacillus helveticus* cells appear orange (hybridization by both probes). Other bacteria appear red (hybridized only by Eub338 probe). b Simultaneous use of the probes Lbh1 (green label) and St4 (red label). *Lactobacillus helveticus* cells appear green, while *Streptococcus thermophilus* cells are red

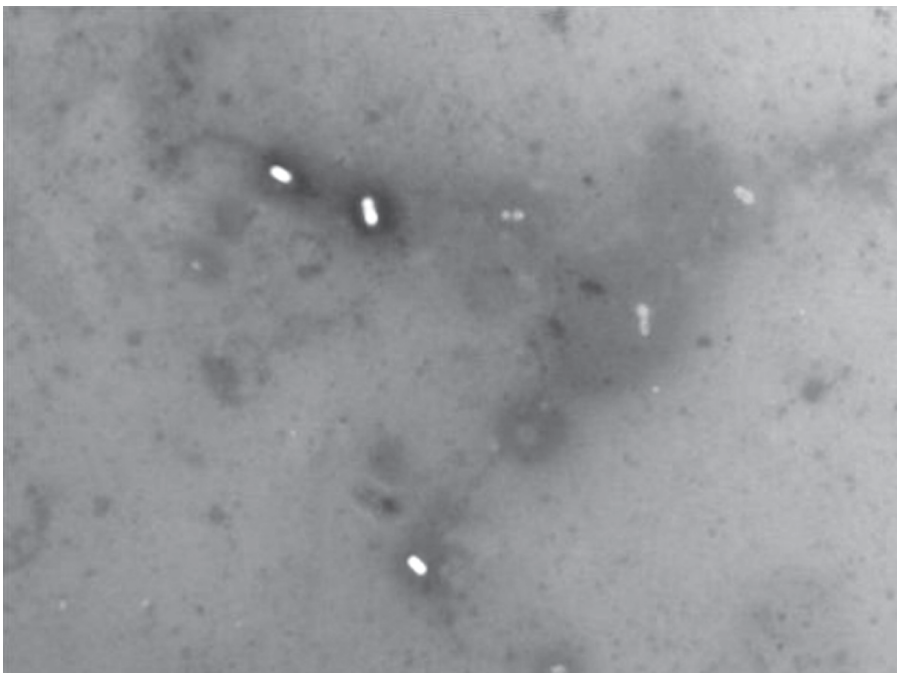

Fig. 2 FISH analysis of experimental cheese inoculated with 106 cells/g of *Escherichia coli*. The micrographs show the microscopic field after FISH analysis of the cheese suspension with the species-specific probe Eco1482 (see Table 1). Brightest cells: hybridized *E. coli* cells. Darkest cells: unhybridized lactic acid bacteria.

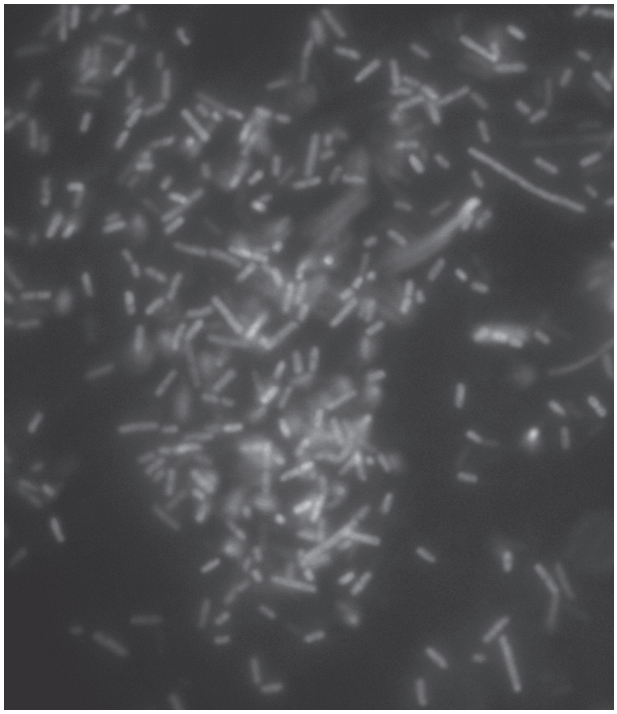

Fig. 3 FISH of a cheese section performed with the eubacterial probe Eub338

3.1.8 Troubleshooting

Accuracy and reliability of FISH

Accuracy and reliability of FISH is highly dependent on the specificity of the oligonucleotide probe, which is strictly correlated to the stringency of protocols. Both formamide and sodium chloride are used to adjust the stringency conditions of hybridization buffer and wash solution respectively. The choice of the opportune concentration of these chemicals, in necessary for proper annealing of the oligonucleotide probes to the target site. In particular, formamide decreases the melting temperature by weakening the hydrogen bonds, thus enabling lower temperatures to be used with high stringency. The more concentrated is the formamide, the more stringent is the hybridization, and the highest the specificity of the hybridization. However, further addition leads to a drastic drop of bound probe and signal intensity (Manz et al. 1992; Bond and Banfield 2001). Even

hybridization washes may be conducted under more stringent conditions so as to remove excess probe and to disrupt all duplexes other than those between very closely related sequences. The stringency of the washing buffer can be regulated by varying the concentration of salt instead of by using formamide, thus reducing the amount of toxic waste (Lathe 1985). Increasing the concentration of NaCl enhances the stability of mismatched heteroduplexes, thus lowering salt concentration encourage dissociation (denaturation) of mismatched heteroduplexes and give higher washing stringency.

Cell permeabilization conditions

Technical problems could arise when optimizing cell permeabilization conditions, which are affected by different cell growth phases (de Vries et al 2003) and by the simultaneous presence of Gram positive and negative bacteria (Ercolini et al. 2006; Thurnheer et al. 2003) in the studied sample. For this reason, different permeabilization treatments have to be screened in order to figure out the most effective for each single case. For example, an extended duration of lysozyme treatment could give the hybridization of all cells but, as drawback, the cells could often display a diffuse appearance suggesting loss of cell structure and leakage of cell content including rRNA.

Probe penetration

Sometimes penetration of probes into bacteria can be variable and insufficient depending on cell wall characteristics. This problem could be overcome trough the use of peptide nucleic acids (PNAs) (Stender et al. 2001) which hybridize to target nucleic acid molecules more rapidly and with higher affinity and specificity compared to DNA probes (Jain 2004). In fact, PNA molecules are uncharged DNA analogues that bind to nucleic acids much more strong that

oligonucleotides because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar-phosphate backbone of the target molecule (Ray et al. 2000).

Use of more than one probe

When more than one probe is used for the same specimen, they have to be mixed in the hybridization buffer at suitable ratios up to 10 ng (Ercolini et al. 2003b).

FISH-washing

Washing steps have to be done carefully, in order to avoid the lost of cells or sample. For this reason, the first aliquots of washing buffer or water must be poured softly at the edge of the spots or the food section, since they are completely covered.

Evaluation and autofluorescence

To overcome the problem of autofluorescence of food matrix and of microorganisms themselves, use of narrow-band filter sets, monochromators, and signal amplification systems are recommended (Sorensen et al. 1997; Shonhuber et al. 1999).

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3.2 Yeast viability and fermentation activities in artisanal pilsner beer

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International Journal of Food Microbiology. Submitted

3.2.1 Abstract

The impact of yeasts on the production and quality of beer is intimately linked to their viability and fermentation activities from the first pitching throughout the subsequent cycles of fermentation. The production of an artisanal beer, made by brewers using traditional practices on a small scale, is founded on the empirical adjustment of parameters.

Aim of this study was to monitor yeast viability during different stages of artisanal beer productions and to correlate it with fermentation dynamics in order to increase process standardization and to maintain the quality of final product. Yeast viability was evaluated during seven fermentation cycles of the artisanal pilsner beer VIÆMILIA, single or double batch brewed. Further, fermentation activities were monitored determining glucose and maltose consumption and ethanol production. We observed that, standardization of procedures and parameters from raw materials throughout all fermentation batches, plays a key role in producing a more uniform and with constant features product. The total number of cells was in the range of 10^8 to 10^9 cell/ml for each batch of fermentation, but yeast viability was found to be variable according to the selected empirical parameters of brewing. In both single and double batch brewed trials, yeast inoculated with higher viability performed better in fermentation. For the single batch brewed trials, when the viable cells percentage in the inoculum was higher than 50%, we observed a faster sugar consumption. For the double batch brewed trials, an higher percentage

of viable cells in the inoculum, corresponded to a greater consumption of sugars before the top up and to a faster reaching of ethanol production and yield stability.

Studying yeast viability and fermentation performance expressed as sugar up-take and ethanol production, here we provide evidence to suggest that yeast viability should be taken into account for improving process standardization and maintaining the quality of the final product.

Keywords: artisanal beer, yeast viability, fermentation activities

3.2.2 Introduction

Since the first records of beer production by mankind, many changes have been made that resulted in the modern beer-brewing process and many beer styles have developed over time (Lodolo et al. 2008). An alternative attitude and approach to brewing of flexibility, adaptability, experimentation and customer service, is represented by microbreweries. Micro or craft breweries have adopted a different marketing strategy than large, mass-market breweries, offering products that compete on the basis of quality and diversity, instead of low price and advertising. Despite all the changes, brewing yeast is always the main character in beer brewing (Lodolo et al. 2008). The impact of yeasts on the production and quality of beer is intimately linked to their viability and fermentation activities from the first pitching throughout the subsequent cycles of fermentation. Brewing is in fact unique within alcoholic beverage industry in that the yeast is not discarded after use but is maintained and reused a number of times (Powell et al. 2003). The physiological state of the inoculum coupled with the conditions established at pitch influence subsequent patterns of growth. Predicting the capacity of pitching yeast towards its subsequent fermentation performance is of key importance (Guido et

al. 2003). In particular, inoculating worts with the correct number of yeast cells at the appropriate viability state is critical for consistent fermentation performance (Cahill et al. 1999). While yeasts cell number is routinely determined, yeast viability is a key parameter that should be measured more frequently. Furthermore, as yeast progresses through the brewing cycle of storage, pitching, fermentation, cropping and storage, it is subject to a number of stresses, thus, viability tests can be used not only to achieve a desired pitching rate but also to constantly check the quality of yeast that could affect subsequent fermentation performance. In fact, a key feature of any good brewhouse is reproducibility. Consumers will appreciate some variation in the beer in the interest of presumed authenticity but will not tolerate a taste too far from the one he likes and he has chosen a particular brand for. Whether the pitching yeast is brand new or derived from a previous fermentation, yeasts viability has been proven to play a key role in maintaining the reproducibility between different batches (Boyd et al. 2003, Cahill et al. 1999). Beer quality is strongly influenced by the biochemical performance of yeast during fermentation. Rate and efficiency of sugars utilization and ethanol yield, and consequently the character of the final product, can be affected by many factors, among which the yeast viability (Gibson et al. 2007). Cell viability is usually defined as the ability of a cell to reproduce and to form colony. It is conventionally measured using the laborious and time consuming culturing methods which are too slow to be of practical use. Moreover, colony counting does not reliably report on the metabolic capacity of slow-growing or non dividing cells missing out the physiological processes other than those involved in cell division that can be of considerable practical importance (Millard et al. 1997). Other that being the ability of cells to grow, reproduce and interact with their immediate environment (Smart et al. 1999), viability

is therefore ultimately linked to vitality, that is a measure of activity (D'Amore 1992), fermentation performance (Boulton 1991) and the capacity of recover from physiological stress (Smart 1996). Aim of this study was to monitor yeast viability during different stages of different artisanal beer productions and to correlate it with fermentation dynamics in order to increase process standardization and to maintain the quality of final product.

3.2.3 Materials and methods

Brewing

The samples analyzed in this study were obtained from different batches of VIÆMILIA, an artisanal pilsner beer produced in Roncole Verdi in the province of Parma (Italy). VIÆMILIA is brewed using only barley malts, water, hops (according to German purity law *Reinheitsgebot* of 1516) and yeast.

Wort production

Wort production took place in the brewhouse and started with mashing operations. Mashing method of VIÆMILIA followed the multi-step infusion system, which consists in mixing water with milled malts (mash-in) and heat the mash at different steps: 20 minutes at 52°C, 20 minutes at 66°C and 25 minutes at 72°C. After mashing, unhopped wort was separated from the undissolved solids, the spent grains or draff. Infusion mashing was carried out for 20 minutes in the mash tun. Then wort (liquid) was withdrawn from the mash and filtered through the bed of grist particles. When bright the wort was moved directly to a copper to be boiled with hops for 100 minutes. After boiling hop fragments were separated in a whirlpool tank . After cooling at 11,5 °C wort was aerated to provide oxygen for the yeast at

initial stages of fermentation and transferred to fermentation tank where yeast pitching took place.

Fermentation

Fermentations were led in cycles. For the first cycle a pure colture (W-34/70, purchased from Uberti SRL, Venice, Italy) was used while for next cycles yeast collected from the bottom of fermenter was repitched into another batch of wort. According to production needs, repitching was done either the same day or after yeast storage up to three days at 4 °C. Primary fermentation was lead at 11,5 °C to a final gravity of 2,5 °P (1010 g/L), then green beer was racked to maturation tank where was slowly cooled to 2,5 °C and held at this temperature for three weeks. During the lagering dried hop cones were added to the beer to obtain a further extraction of the finest and more volatile aromas from hops. After dry hopping, mature beer (three weeks) was artificially carbonated and packed. VIÆMILIA was brewed with either single batch or double batch fermentation, when sterile cooled wort was added to the beer in fermentation without giving any aeration neither any further yeast inoculum.

Sampling

Seven fermentation cycles were monitored. Samples were obtained at 0, 16, 40, 64, 88, 112, 136 and 160 hours from single batch fermentations (a, b, e), and at 0, 16, 21, 24, 40, 64, 88, 112, 136 and 160 hours from double batch fermentations (c, d, f, g). Time 0 is referred to inoculum before pitching. One more sample was collected after the top up (23 hours) for fermentation activities analysis.

Yeast viability

Aliquots of 1 ml of each sample were centrifuged for 5 min at 10000 rpm, in order to separate cells from wort. Yeast viability has been determined by using the Live/Dead Yeast viability kit (Molecular

Probes, Eugene, Oregon). This kit contains a novel two-colour fluorescent probe for yeast viability, FUN® 1, which exploits normal endogenous biochemical processing mechanisms that appear to be well conserved among different species of yeast and other fungi (Millard et al. 1997). The conversion of FUN® 1 cell stain from a diffusely distributed pool of green fluorescent intracellular stain to a compact form consisting of cylindrical fluorescent intravacuolar structures (CIVS), requires both plasma membrane integrity and metabolic capability. Only metabolically active cells are marked clearly with fluorescent intravacuolar structures, while dead cells exhibit extremely bright, diffuse, green-yellow fluorescence. Cells with intact membranes but with little or no metabolic activity have diffuse green cytoplasmic fluorescence and lack fluorescent intravacuolar bodies. After collection, cells were washed in sterile Millipore water and resuspended in 0.50 mL of sterile GH (2% glucose in 10mM Na-Hepes pH 7.2). 2.0 µL of 10 mM FUN-1 stock was added to each microcentrifuge tube and the cells were vortexed. Cells were then allowed to incubate with agitation at 30°C for 30 min. Cells were then spun down and washed twice in sterile Millipore water. Stained yeast cells were examined under a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp. Nikon filter set B2A FITC was used for FUN1® (excitation wavelength, 450-490 nm; emission wavelength, 500–520 nm). Nikon filter set G-2E/Cwas used for FUN1® converted (excitation wavelength, 540/25 nm; emission wavelength, 605/55 nm). At least 10 fields with approximately 20 cells in each were counted for each sample at a magnification of ×100. For each field, fluorescent cells were counted and the resulting values were averaged. To express counts as cells/ml, the average values were multiplied by 5.21×10^8, a dilution factor to account for the coverslip area (400 mm^2), the area of

each field (0.003072 mm^2), the volume of the sample deposited onto the slide (100 µl), and the sample dilution. Nis Elements software (version 2.10 Nikon) was used for image analysis and cell counts. The software allowed separated images of yeasts cells with or without CIVS to be superimposed.

Fermentation activities

Fermentation activities were monitored determining sugar consumption and ethanol production. Maltose and Glucose consumption was assayed with Maltose/Sucrose/D-Glucose UV-method and D-Glucose UV method (Boehringer Mannheim, R-Biopharm, Darmstadt, Germany), respectively. Ethanol production was determined with Ethanol UV-method (Boehringer Mannheim, R-Biopharm, Darmstadt, Germany). After centrifugation and appropriate dilution, samples were analyzed according to manufacturer instructions. Glucose, maltose and ethanol amount are measured by means of their light absorbance at 340 nm. Then, results were expressed as g/L, according to the general equation for calculating the concentrations provided on the kit technical sheet.

3.2.4 Results and Discussion

To ensure effective fermentation and high quality beer with constant features, appropriate brewing conditions and yeast viability maintenance must be aimed. The response to the numerous stresses to which the yeast is exposed, from fermentation of wort and through handling between fermentations, is crucial for beer production but also for maintaining the fermentation fitness of yeast for subsequent fermentations (Gibson et al. 2007). By selecting a yeast in a good physiological state, a rapid fermentation may be achieved, in order to produce a beer with the maximum attainable ethanol content, consistent with the overall flavour balance of the product (Guido et al.

2004). Studying yeast viability and fermentation performance expressed as sugar up-take and ethanol production, here we provide evidence to suggest that yeast viability should be taken into account for improving process standardization and maintaining the quality of the final product. Dynamics of yeast viability are shown in Fig. 1.

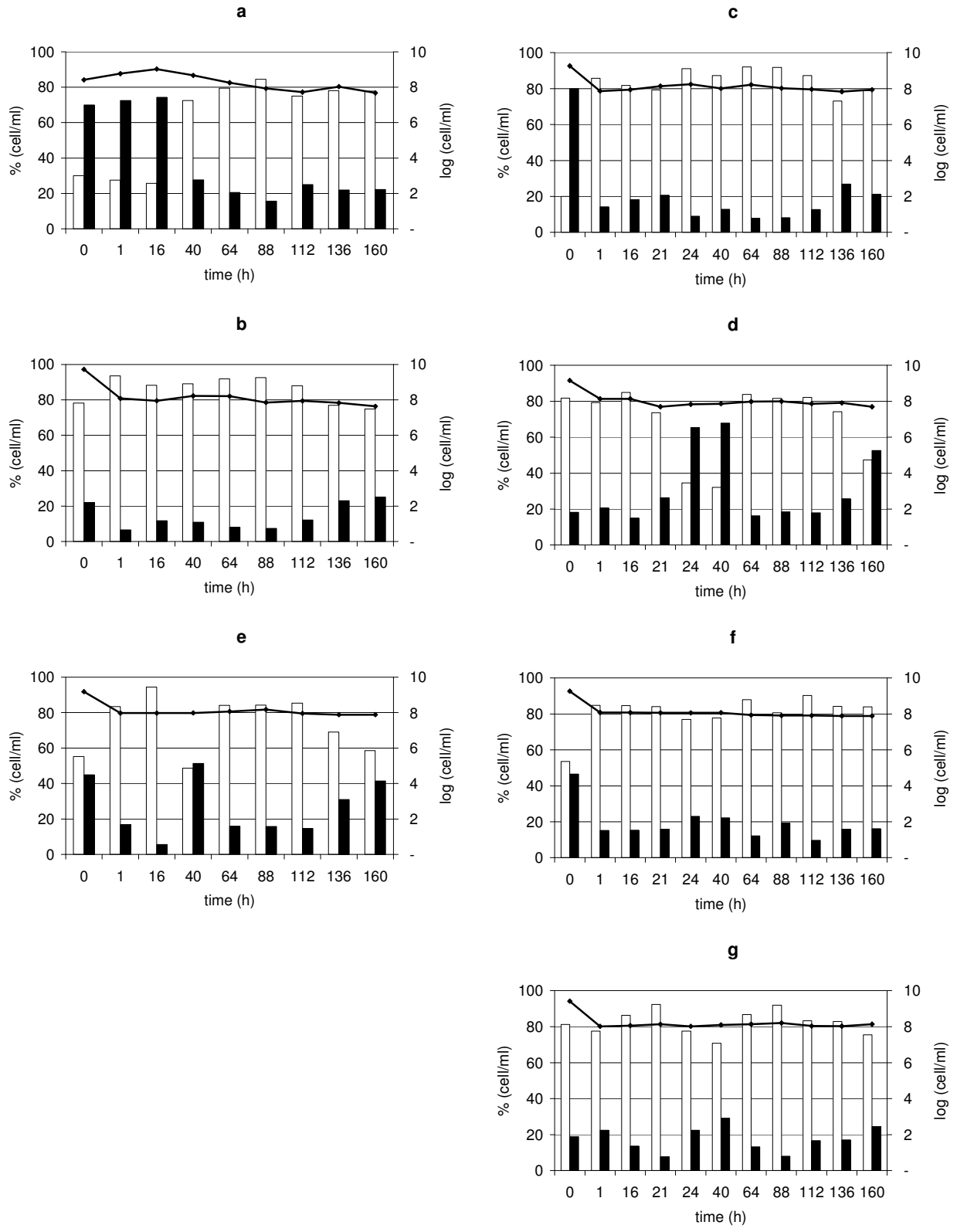

Figure 1. Yeast viability in the 7 considered batches. a, b, e are single batch brewing; c, d, f, g are double batch brewing. □ percentage of viable cells; ■ percentage of non viable cells.

According with general breweries protocols (Briggs et al. 2004), the initial yeast concentration was relatively high and the subsequent growth extent modest. The total number of cells was in the range of 10^8 to 10^9 cell/ml for each batch of fermentation. Yeast viability was found to be variable according to the selected empirical parameters of brewing. Viability was low at the beginning of the first fermentation, when dry inoculum had been used (trial a), confirming that the culture must revert to its fully functional state and adapt to its new environment before it can perform efficiently (Finn and Stewart, 2002). Adaptation of cells to brewing conditions resulted in higher viability, well maintained all the batch of fermentation long. The subsequent cycles yeast viability has been affected by yeast handling before re-pitching. Viability rate was higher when yeast was directly reused after the previous fermentation end (trials b, d), while an increased number of damaged cells was found when yeast slurry was stored at 4°C after cropping (Fig. 1 trial c). These results are in agreement with Cahill et al. (1999) who affirmed that during prolonged storage, the quality of the yeast deteriorates and the percentage of viability decreases accordingly. As observed in previous works (Guido et al. 2004), yeast showed to be able to recover its viability throughout fermentation. However, viability has a greater importance in the earlier phases of fermentation, when the fermentative capacity is not due to the yeast growth or multiplication, but instead to the intense metabolic activity and, thus, to the viability of the pitched yeast (Guido et al. 2004).

It is known that, during fermentation, the yeast sugar up-take follows the route of simplest sugars like glucose first, followed in increasing order of complexity by disaccharides (maltose) and trisaccharides (maltotriose) excluding maltotetraose and other dextrins (Lodolo et al. 2008). For the single batch brewed trials, when the viable cells percentage in the inoculum was higher than 50%, (Fig. 2 trials b, e) we

observed a faster sugar consumption: e.g. 40 hours for glucose and 64 hours for maltose consumption in trial b versus 64 and 136 hours respectively in trial a. Faster sugar consumption and the resultant gradual ethanol production, led to a 24 hour shorter production process (Fig 2). For the double batch brewed trials, an higher percentage of viable cells (Fig. 2 trials d) in the inoculum corresponded to a greater consumption of sugars before the top up and to a faster reaching of ethanol production and yield stability. However, although the most of pitched cells were viable, sugars up-take in trials f and g was lower than in trials d (Fig. 2), as a probable consequence of the approaching of serial repitching termination.

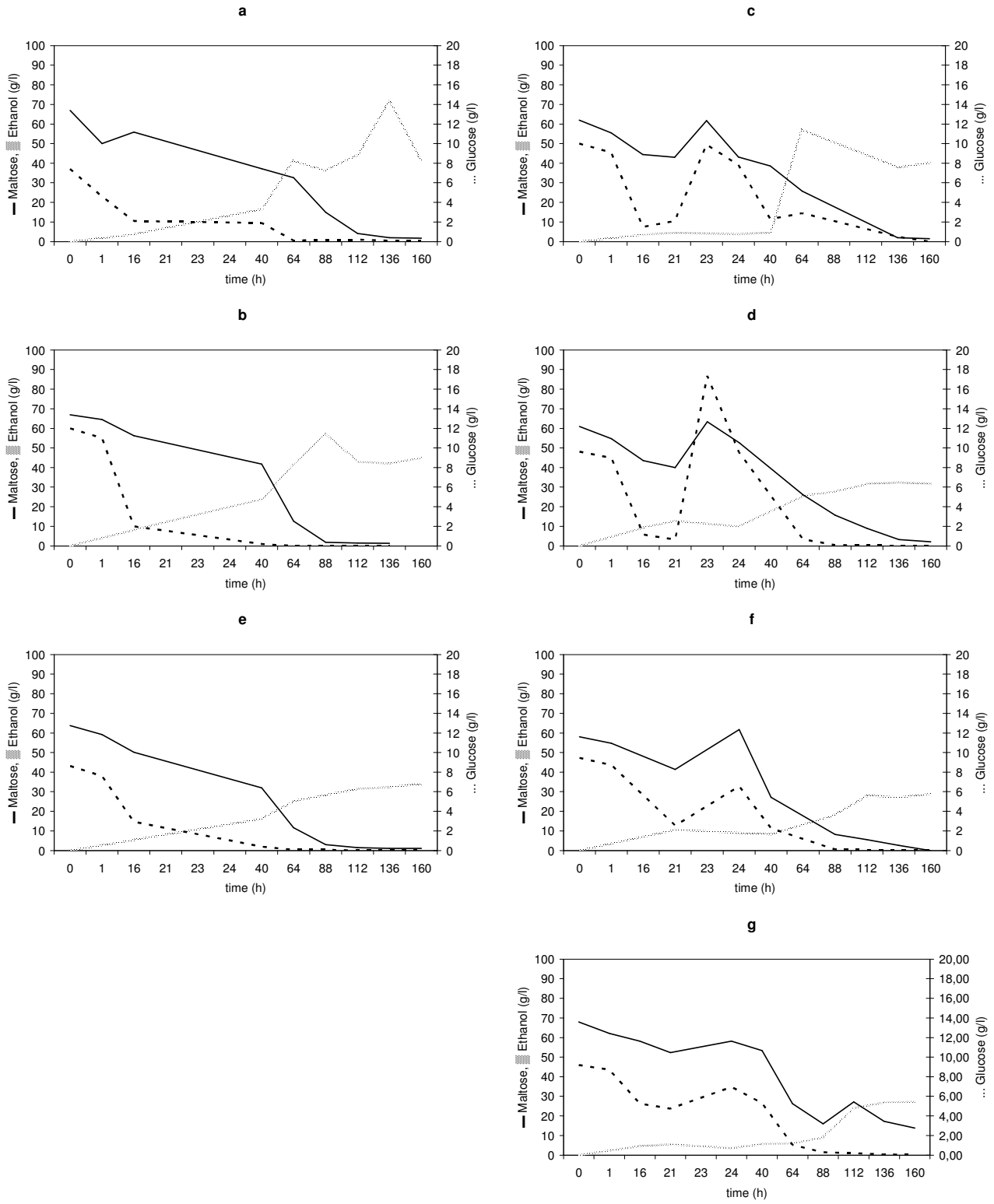

Figure 2. Glucose [---] and maltose [▬] consumption; ethanol [▒] production in the seven considered batch. a, b, e are single batch brewing; c, d, f, g are double batches brewing

In agreement with Powell et al. (2003), this seems to suggest that towards the end of times of yeast reuse, fermentation capability is mainly an outcome of cell age rather than a viability defect. Furthermore, it is known that serial repitching cycles cause changes in the activity of yeast cells, which are responsible for prolonged fermentation and strong flavour beer production (Kobayashi et al 2006). Anyhow, in both single batch and double bacth brewed trials, yeast inoculated with higher viability performed better in fermentation. More viable yeast in batches b and d fermented more rapidly early in the fermentations. Worts inoculated with dry yeast (Trial a) or with stored cropped yeast (Trials c, e, f) have been fermented more slowly and with a less constant ethanol increase. Even though the final ethanol yield was not affected by early fermentation rates, pitching yeast with a lower viability percentage caused a delay in fermentation start (Trials c, f). Longer fermentation can cause a "bottle neck" in the process, which is of particular significance on a small artisanal brewery, that is not able to solve the problem further increasing size or number of fermenters (Lodolo et al. 2008). An extended fermentation time has a direct impact on plant efficiency, with subsequent financial implications (Powell et al. 2003). Furthermore, if the performance of the fermentation does not fall within the normal range in terms of duration and extent of yeast growth, the resultant beer could not be within specification. Although chemical analysis have non been undertaken in this study, Guido et al. (2004) showed that yeast physiological conditions affect several quality parameters, such as the concentration of beer volatile esters and higher alcohols and the beer flavour stability.

Beer is hard to spoil and has a remarkable microbiological stability because it is an unfavorable medium for many microorganisms. This is due to the presence of ethanol, hop bitter compounds, the high

content of carbondioxide, the low pH, the extremely reduced content of oxygen and the presence of only traces of nutritive substances such as glucose, maltose and maltotriose. In fact, this latter carbon sources are usually almost completely metabolized by yeast during a fast and correct fermentation (Sakamoto et al 2003). If the carbon sources are not completely depleted, they remain available for spoilage microorganisms, like lactic acid bacteria, that could develop more easily.

Despite brewing parameters in an artisanal brewery are often bound to empirical factors such as fermenter availability and consumer demand, brewer should keep on chasing reproducibility and process standardization in order to guarantee a constant high quality of the final product. In this study, we used viability measurement to monitor yeast activity through pitching and reuse. Periodical evaluation of this parameter, could be the starting point for an effecting yeast management program, in order to maintain or even improve the quality properties of the final beer.

Acknowledgements

The authors are grateful to Dr. Francesca Zerlotti and Roberto Manotti for their technical contribution.

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3.3 Dynamics of Whole and Lysed Bacterial Cells during Parmigiano-Reggiano Cheese Production and Ripening

Monica Gatti, Juliano De Dea Lindner, Angela De Lorentiis, Benedetta Bottari, Marcela Santarelli, Valentina Bernini, and Erasmo Neviani, Applied and Environmental Microbiology, 2008, p. 6161-6167, Vol. 74, No. 19

3.3.1 Abstract

The microbial succession during a Parmigiano Reggiano cheesemaking was monitored by length heterogeneity PCR (LH-PCR) considering the intact and lysed cells at different stages of cheese production and ripening. When starter species underwent autolysis, species coming from milk were able to grow. For the first time LH-PCR technique was applied to study a fermented food

Parmigiano Reggiano (PR) is a Protected Designation of Origin cheese, produced in specific areas of Northern Italy. It is a hard-textured, cooked, and long ripened cheese made from raw cow's milk supplemented with natural whey starter rich in thermophilic lactic acid bacteria (LAB). Microbiological features of PR have been studied isolating on traditional growth media a great number of strains from a large amount of samples representative of the production and of the earlier and advanced stages of ripening (3, 4). Other studies were focused on the biodiversity of different strains of *Lactobacillus helveticus* isolated from natural whey cultures (9, 10). These previous studies showed the microbial biodiversity of PR but not its real

succession during the production and ripening, and the use of culture-dependent methods could have underestimated the less abundant components of microflora equally important for cheese ripening and flavor development (28). Thus the microbial ecology of PR appears still not completely understood. From this perspective, a detailed knowledge of LAB dynamics during manufacturing and ripening stages is necessary for a deeper insight into the complex process which bring to the outcome of this appreciate cheese.

In the present study, LH-PCR was used to monitor the microbial dynamics during 24 months of PR ripening for both the entire and lysed cells. The availability of PR twin wheels, allowed us to have samples representative of the subsequent stages of the same cheese making process.

In our opinion the new findings shown in this paper, contribute to a better understanding of microbial dynamics in a complex fermented ecosystem.

3.3.2 Cheesemaking.

A unique cheese factory was selected for its equipments and technological conditions in order to obtain a suitable number of PR twin wheels. The same milk was distributed in equal volume in eight vats and was worked according to PR production standard.

Thirty-four liters of natural whey starter were added in each vats containing 1070 l of milk (525 l of partially skimmed evening milk and 545 l of whole morning milk), bringing the pH of the mixture to 6.20. After cheesemaking and molding, the wheels, two from each vat, were held for 3 days and frequently turned to enable the complete whey drainage. They were then salted by immersion in brine at 300 g NaCl·l^{-1} for 23 days. Ripening was held in aging rooms with 85% relative humidity and about 18°C temperature for 24 months.

3.3.3 Cheese sampling and bacterial recovering.

Samples were collected from milk to 24 months ripened cheese. Aliquots of the total mass of raw milk, whey starter, milk plus whey starter in vat and curd at the vat extraction were sampled. For each of the following 14 samples the whole wheel was sacrificed: curd after 6, 12 and 48 h from extraction, salted cheese and cheese at different stages of ripening (2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 months). The remaining two wheels will be object of further researches. The wheels were lengthwise cut along vertical axis and a central 10 cm thickness cheese section was obtained. Internal and external (far 0.5 cm from cheese crust) portions were obtained from these cheese sections. In order to have a sample representative of the whole wheel, the two portions were mixed in equal weight and grated before the analysis. Raw milk, whey starter and milk plus whey starter in vat were 10-fold

serial diluted in 50 mM sodium citrate buffer pH 7.0, while 10 g of curd and cheese samples were first homogenized with 90 ml of sodium citrate buffer for 3 min in a blender (Stomacher 400, Seward, UK).

In order to recover an heterogeneous bacterial population, different types of nutritional media and the following time/temperature conditions of incubation were used: MRS agar pH 5.4 (Oxoid Spa, Italy) at 42°C for 48 h and 25°C for 72 h; M17-SSW (7) at 42°C for 42 h and 25°C for 72 h; Whey agar medium (WAM) (9) at 42°C for 48 h; Curd agar medium (CURDAM) (16) and Cheese agar medium (CAM) (6, 16) at 42°C for 72 h, all under anaerobic conditions.

LAB originate from raw milk were lower than 4 log CFU ml^{-1}. The natural whey starter was characterized by a high number of thermophilic lactobacilli (Table 1). Total cultivable microbial population was high and not variable during the first six months. After 9/10 months of ripening a substantial decrease of cultivable bacteria was observed. Similar growth trends were observed in previous studies (3, 4) even if the results are not easily comparable because different media have been used.

TABLE 1. Bacterial counts in different media of cultivable lactic acid thermophilic and mesophilic bacteria during PR cheese production and ripening

	M17-SSW		MRS pH 5.4		WAM	CURDAM	CAM
Samples	25°C	42°C	25°C	42°C	42°C	42°C	42°C
Whey Starter	n.d	4,89	n.d	7,00	8,53	7,00	1,00
Caseification Milk	3,61	3,89	2,13	1,95	2,43	3,17	3,23
Curd at vat extraction	3,52	5,05	3,03	6,36	7,00	4,00	2,96
Curd 6 h	3,66	5,55	2,35	7,25	8,17	7,53	3,31
Curd 12 h	4,76	4,87	3,05	6,97	7,89	6,93	3,70
Curd 48 h	4,85	4,34	2,26	5,70	7,67	7,19	3,11
Salted cheese	5,65	5,94	5,64	5,63	6,48	4,15	5,06
Cheese 2 months	6,40	6,21	6,18	6,77	7,21	7,07	7,18
Cheese 3 months	4,86	4,18	6,75	7,29	6,54	7,13	7,08
Cheese 4 months	5,00	4,78	7,08	7,08	6,91	6,15	6,88
Cheese 6 months	4,15	3,74	7,10	6,85	7,02	6,35	7,19
Cheese 8 months	4,91	4,90	7,56	6,65	6,84	5,17	6,91
Cheese 10 months	4,57	4,17	6,17	6,28	5,94	5,10	6,42
Cheese 12 months	4,77	4,54	5,83	5,93	5,51	5,39	6,06
Cheese 16 months	4,30	4,00	5,13	5,30	5,40	4,60	5,11
Cheese 20 months	3,77	3,00	3,95	4,34	4,00	3,18	3,65

3.3.4 Strains isolation, clustering and set-up of an LH-PCR database.

To set-up an appropriate LH-PCR database, a total of 187 strains were isolated from the five media used for all 18 stages of PR manufacturing and ripening. Genomic DNA of the isolated strains was extracted from overnight cultures by a Chelex-based procedure according to Rossetti and Giraffa (25). DNA amplification and sequencing were performed as previously described (12), and each sequence obtained was checked

manually and searched for sequence homology using Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

With the aim of clustering strains with genotypic relatedness, all species identified by 16S-rRNA gene sequencing were fingerprinted by RAPD-PCR as previously described (25). Clustering of the patterns was achieved through the unweighted pair group method and employing arithmetic averages (UPGMA) using the BioNumerics™ software (package version 3.0; Applied Maths BVBA, Belgium). Calculation of similarity of the PCR fingerprinting profiles was based on the Pearson product-moment correlation coefficient. Strains with similarity coefficient higher than 80% in the dendrogram were considered belonging to the same biotype.

The dendrogram revealed 35 clusters showing a high biodiversity among strains and species present in a complex system like PR cheese (Fig. 1).

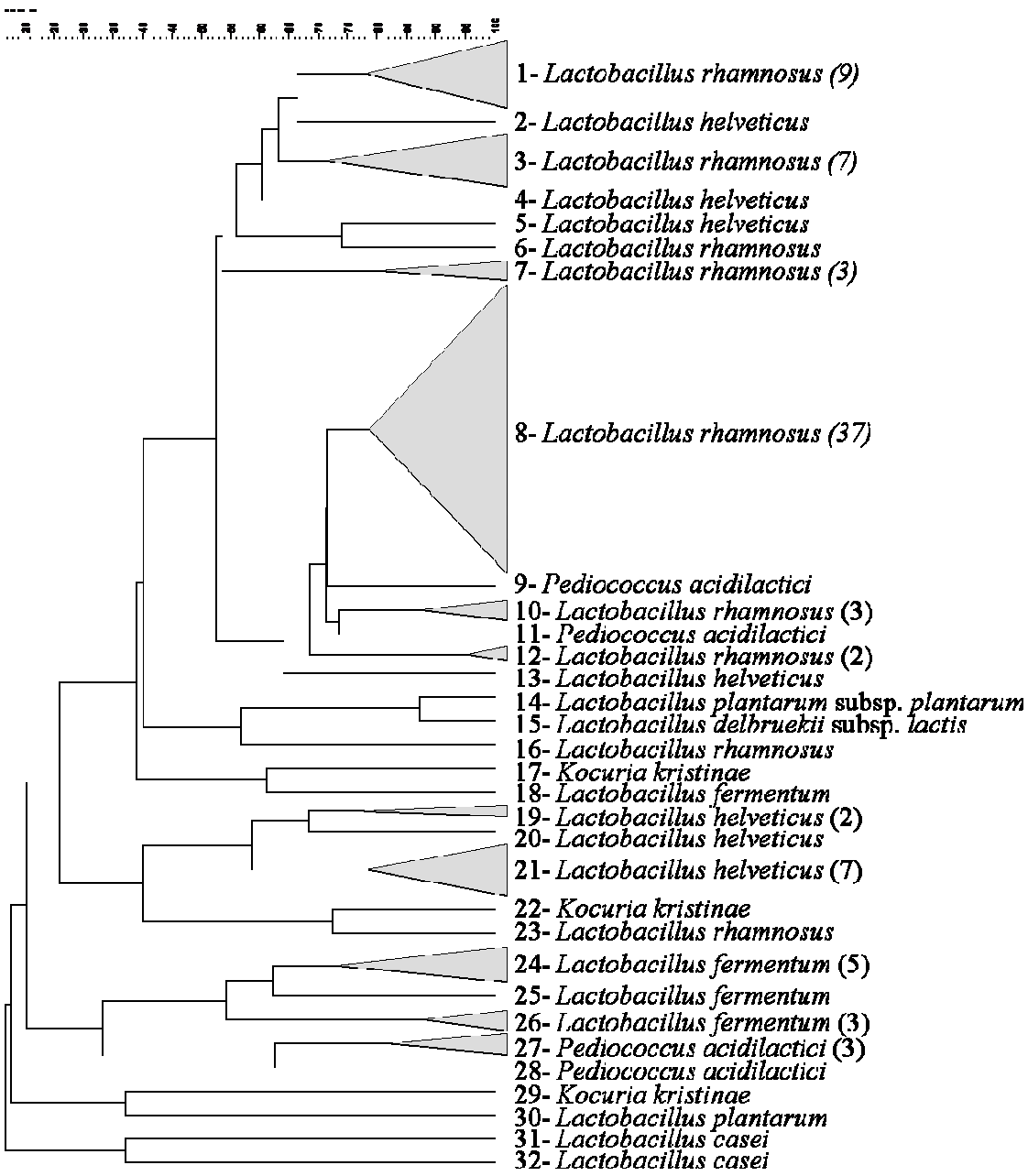

FIG. 1. Cluster analysis of RAPD-PCR patterns obtained with a primer M13 of strains isolated from samples. Clustering was performed by an UPGMA of Pearsons product moment correlation coefficient (expressed as a percentage). Numbers from 1 to 32 report the RAPD-PCR clusters or branches. The number of strains isolated for each cluster is indicated between brackets.

Thirteen strains, belonging to eight different species or belonging to the same species but with a different RAPD-PCR profile and coming from different samples, were chosen to generate the database. Moreover, six type strains of other species isolated from PR cheese but not found in this study were included (Table 2).

TABLE 2. LH-PCR fragment length database of 14 strains isolated from cheese samples belonging to different RAPD-PCR clusters and six type strains

Strain n°	RAPD-PCR cluster or branch	Number of isolates †	Origin	16S-rRNA Blast closest relative or Type strain	Acession number‡	% match	Fragment size* (bp)
750	29	1	Curd 12 h	*Kocuria kristinae*	AF375912	100	310
714	15	1	Milk	*Lactobacillus delbrueckii* subsp. *lactis*	AB289095	99	330 (284)
779	2	1	Curd 48 h	*Lactobacillus helveticus*	AB008210	100	334
776	21	7	Curd 6 h	*Lactobacillus helveticus*	AB008210	98	334
1056	31	1	Cheese 6 months	*Lactobacillus casei*	AJ558112	100	335
1247	32	1	Curd 12 h	*Lactobacillus casei*	AB008205	100	335
830	7	3	Salted cheese	*Lactobacillus rhamnosus*	EU184020	100	336 (290)
1489	8	37	Cheese 20 months	*Lactobacillus rhamnosus*	EF533991	99	336 (290)
710	14	1	Curd 48 h	*Lactobacillus plantarum* subsp. *plantarum*	EF577047	99	337 (290, 308)
1026	18	1	Cheese 6 months	*Lactobacillus fermentum*	AF477498	99	342, 344

730	26	3	Curd 6 h	*Lactobacillus fermentum*	EU221276	99	342, 344
1466	9	1	Cheese 20 months	*Pediococcus acidilactici*	EU147316	99	345
805	27	3	Whey starter	*Pediococcus acidilactici*	EU147316	100	345
Type LMG 6897	/	/	/	*Lactococcus lactis* subsp. *cremoris*	/	/	318 (272)
Type LMG 6896	/	/	/	*Streptococcus thermophilus*	/	/	319 (273)
Type LMG 6890	/	/	/	*Lactococcus lactis* subsp. l*actis*	/	/	318 (272, 291)
Type LMG 11423	/	/	/	*Enterococcus faecium*	/	/	328, 331 (284, 301, 303)
Type LMG 7937	/	/	/	*Enterococcus faecalis*	/	/	329 (283, 299)
Type LMG 6901	/	/	/	*Lactobacillus delbrueckii* subsp. *bulgaricus*	/	/	330
Type LMG 11457	/	/	/	*Lactobacillus parabuchneri*	/	/	345 (255, 299)

V1, V2 and V3 variable region of the 16S rRNA gene of the twenty strains was analyzed by LH-PCR as previously described (15). Amplicons sizes were determined with GeneMapper software (version

4.0; Applied Biosystems, Foster City, CA, USA) with a threshold of 150 fluorescent units.

The database reports fragment lengths from 310 to 346 bp. LH-PCR fragment sizes of the LAB agree with Lazzi et al. (15). Differently from these authors, other secondary peaks where found. As expected, strains belonging to different clusters, but to the same species, gave the same fragment sizes in base pairs (Table 2).

3.3.5 LH-PCR analysis of cheese samples

To investigate the microbial community evolution in PR cheese, grated cheese (and curd) samples were diluted 1:10 in 50 mM sodium citrate buffer pH 7.0 and homogenized in a blender (Stomacher 400, Seward UK) for 3 min, while raw milk, whey starter and milk plus whey starter in vat samples were directly submitted to the following steps. In order to separate entire bacterial cells from the DNA coming from lysed cells, 1 ml of each sample was filtered on a 0.2-µm filter (Whatman GmbH, Dassel, Germany) to obtain free-cell fraction and another milliliter was non-filtered and directly placed in a 1.5 ml microtube. In order to digest free DNA arising from lysed cells, the non-filtered fraction was treated with 0.14 U μl^{-1} of Amplification Grade DNase I (Sigma-Aldrich, Co., St. Louis, MO, USA) under conditions given by the supplier. The samples were centrifuged at 7,500 rpm for 5 min and the pellets were suspended in 100 µl of pure water with the addition of 20 µl 10×

Reaction buffer (Sigma-Aldrich, Co., St. Louis, MO, USA) and 20 µl Amplification Grade DNase. This mixture was incubated for 1 h at room temperature. DNase was inactivated adding 20 µl of Stop Solution (Sigma-Aldrich, Co., St. Louis, MO, USA) and heating at 70°C for 10 min.

DNA was extracted both from the filtered untreated fractions (lysed cells) and from the non-filtered treated ones (entire cells) by means of Qiagen-DNeasy Blood & Tissue kit (QIAGEN GmbH, Hilden, Germany) as described by the manufacturer. DNA was quantified by measuring absorbance at 260 nm and 280 nm (Spectrophotometer Jasco), diluted up to 20 ng μl^{-1} and stored at -20°C until use. LH-PCR was performed as described for database set up.Fig. 2 and 3 show the LH-PCR profiles obtained from LAB entire and lysed cells during PR cheese manufacturing. Only the electropherograms referred to the most representative samples (Whey starter, Curd 48 h, Salted cheese, 2, 6 and 24 months cheeses) are shown. Curd 6, 12 and 48 h electropherograms were similar. Two, 3 and 4 months cheeses gave similar patterns and any evident differences were found in 6, 8, 10, 12 16 and 20 months cheese samples.

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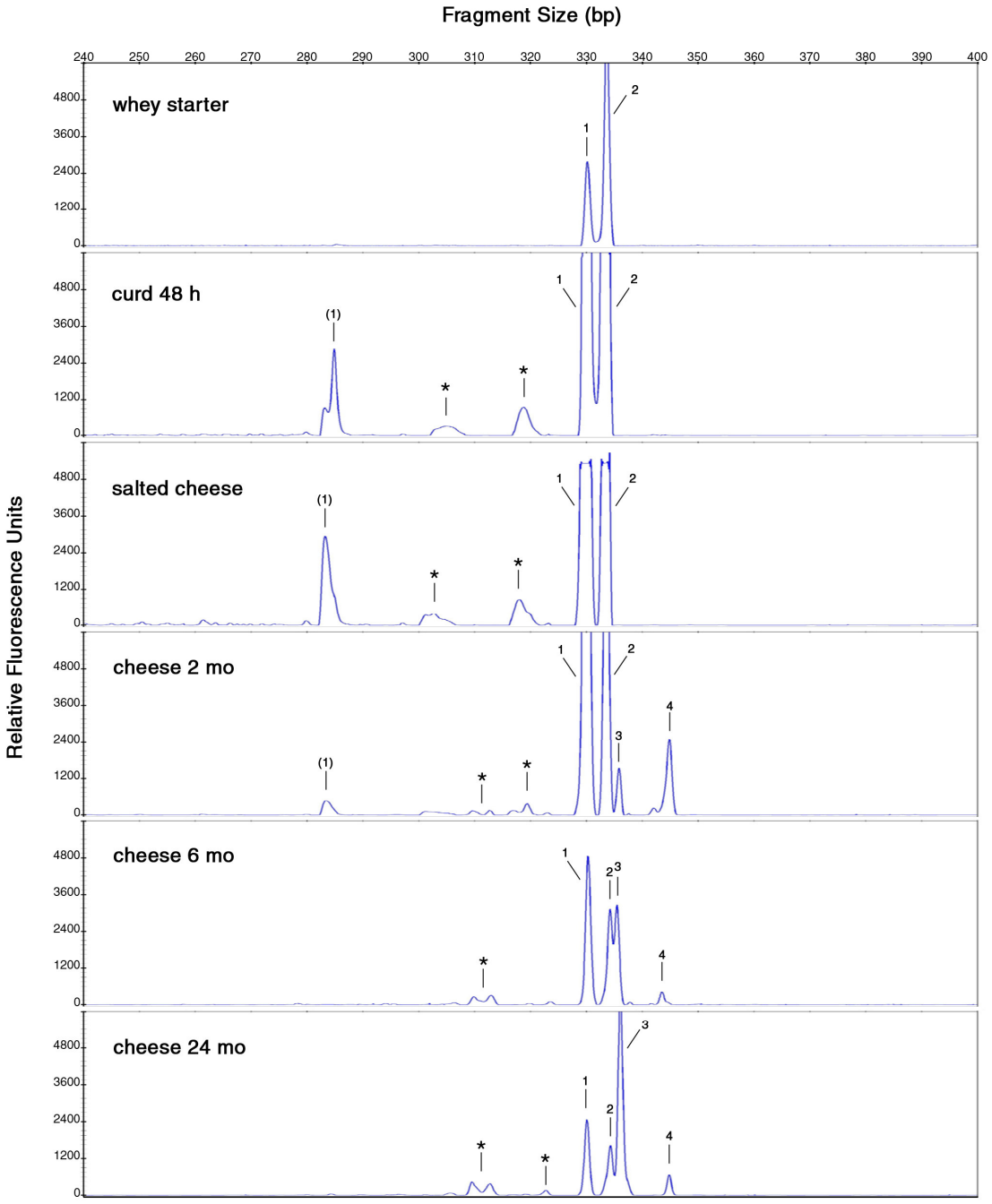

FIG. 2. Length heterogeneity LH-PCR electropherograms of whole cells of LAB communities present in the samples studied at different stages of manufacturing and ripening of PR cheese. The *x* axis shows the peak size in base pairs, and the *y* axis shows the peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to the LH-PCR database (Table 2) as follows: 1, *L. delbrueckii* subsp. lactis or subsp. *bulgaricus*; (1), secondary peak of *L. delbrueckii* subsp. *lactis*; 2, *L. helveticus*; 3, *L. rhamnosus*, *L.* casei, or *L. plantarum*; 4, *L. parabuchneri* or *P. acidilactici*. Unattributed peaks higher than 150 fluorescence units are indicated with the respective base pair sizes.

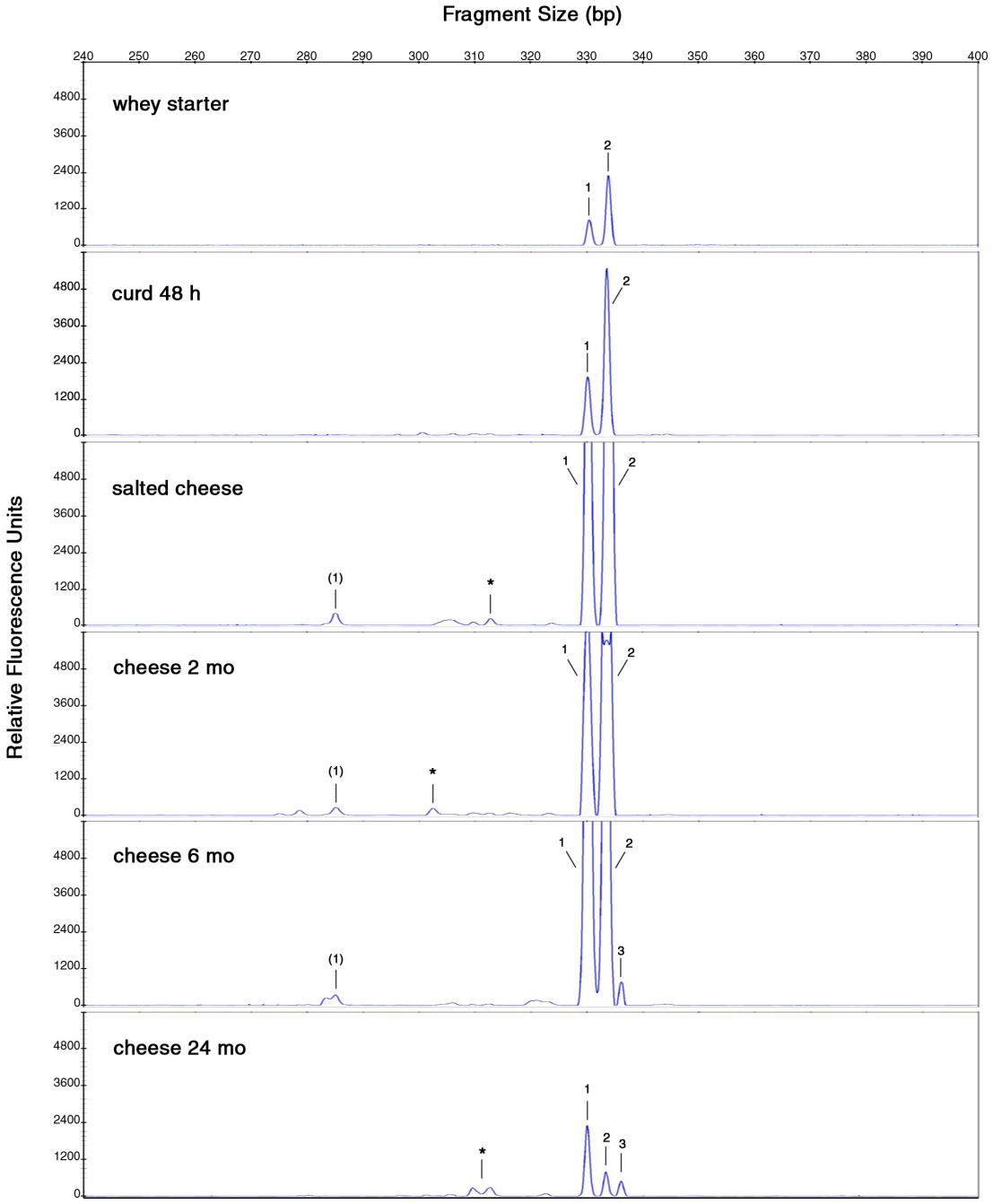

FIG. 3. Length heterogeneity LH-PCR electropherograms of lysed cells of LAB community present in the samples studied at different stages of manufacturing and ripening of Parmigiano Reggiano cheese. The *x* axis shows peak size in base pairs, and the *y* axis shows peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to the LH-PCR database (Table 2) as follows: 1, *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*: (1) Secondary peak of *L. delbrueckii* subsp. *lactis*; 2, *L. helveticus*; 3, *L. rhamnosus* or *L. casei* or *L. plantarum*. Not attribuited peaks are indicated by *.

The different fragment sizes in the LH-PCR profiles were attributed to bacterial species according to the LH-PCR database (Table 2).

The peaks detected in the raw milk entire cells electropherogram were: 330 bp (attributed to *Lactobacillus delbrueckii* subsp. *lactis* or subsp. *bulgaricus* or *Enterococcus faecium* or *Enterococcus faecalis*), 334 bp (*L. helveticus*), 336 bp (*Lactobacillus rhamnosus* or *Lactobacillus plantarum* or *Lactobacillus casei*), and 339 bp (not attributed). The fluorescence intensity was more than 10-fold lower than the average found in the other samples (data not shown). As expected the amount of DNA from filtered raw milk, released from lysed cells, was too low to be amplified (data not shown).

The LH-PCR profile referred to entire LAB cells from natural whey starter showed 2 peaks (Fig. 2), corresponding to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* and *L. helveticus* species. Differently from previous studies on natural whey starter for a similar cheese as Grana Padano (7, 15, 26), *Streptococcus thermophilus* peak (320 □ 1 bp) was not found. Differently from Coppola et al. (4) we have never isolated in our samples *L. delbrueckii* subsp. *bulgaricus* species which, on the other hand, could have been viable but hardly cultivable. Whey starter is expected to have a high percentage of metabolically active cells, however the LH-PCR profile from lysis-released DNA is similar, even if 3-4 fold lower, to the entire cells profile, showing an autolysis phenomenon.

In the 48 h curd entire cells electropherogram, two major peaks attributable to *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* were found. Three other not attributed peaks of minor fluorescence intensity were detected. In the LH-PCR profile from lysis-released DNA of 48 h curd only the two attributable peaks were found.

After one month of brining, salted cheese sample was characterized by a attributable peak pattern of entire cells similar to the previous one. In the lysis-released DNA profile, the attributed peaks were higher than those in the 48 h curd and two unknown peaks appeared. This result could highlight an increase of cells autolysis after the brining.

After 2 months of ripening, entire cells of *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* were found in great amount but no one of these species have been isolated form agar plates (Table 2). These cells could be under quiescent conditions, may be viable but not cultivable and not still lysed. They could be also dead, not yet lysed cells as suggested by fluorescent dyes for live and dead cells assay (data not shown). In addition to *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus,* two other peaks, attributable to *L. rhamnosus* or *L. casei* or *L. plantarum* and to *Lactobacillus parabuchneri* or *Pediococcus acidilactici,* were able to grow and appear only in the electropherogram referred to entire cells.

Electropherogram of entire cells from 6 months cheese showed the same peaks of previous samples but with reduced peaks intensity.

Instead, in lysis-released DNA electropherogram, a peak attributable to *L. rhamnosus* or *L. casei* or *L. plantarum* appeared. This trend persisted without appreciable variations, in the subsequent samples until 24 months of ripening (data not shown).

In the 24 months cheese electropherogram referred to entire cells, the major peak was attributable to *L. rhamnosus* or *L. casei* or *L. plantarum*. Notably, in the lysis-released DNA electropherogram an important decrease of fluorescence intensity was observed. In agreement with Thomas et al. (30) intuition and Nielsen et al. (21), we can suppose that DNA degradation of some microorganisms could be a source of carbon, nitrogen, phosphorus and nucleic acid precursor for less nutritional demanding bacterial cells in a particularly hostile nutritional environment occurring during the last months of PR ripening. Thomas et al. (30) and Williams et al. (32), hypothesized that non-starter LAB could use, as an alternative potential energy source, nucleic acids derived from the autolysis of starter LAB. Even if pure nucleic acids are generally not sufficient as a sole carbon source for bacteria, it has been demonstrated that *Serratia marcescens* and *Escherichia coli* are capable of utilizing DNA exclusively for carbon (21). LH-PCR and RT-LH-PCR have already been successfully used for the analysis of fresh dairy products (15, 26), and to monitor LAB dynamics during maize ensiling (1). As far as we know, this technique has never been used to study a ripened cheese where viable, not viable and lysed

microbial cells are contemporaneously present. Analysis of intact DNA extracted from degraded specimens and tissue sample has become a useful tool for criminal and conservation forensics. In an intriguing review, Nielsen et al. (21) studied the release, breakdown and persistence of bacterial and plant DNA in soil, sediment and water. The recovery of DNA in processed food has already been used for detection and quantification of genetically modified ingredients (17). However, to our knowledge this approach has never been used to evaluate the presence of DNA from lysed cells in fermented food.

Due to the low sensitivity, LH-PCR is not meant to be a quantitative analysis. Otherwise, it gave us the way to follow and be aware of the dynamics of entire and lysed bacterial cells during Parmigiano Reggiano cheese production and ripening, letting us to reach new important findings in the knowledge of this appreciated cheese.

Acknowledgements

This work was partially supported by Emilia Romagna Region (LR28/98), Bologna, Italy, Project "SMEPR Studio e modellazione degli aspetti enzimatici legati alla stagionatura del formaggio Parmigiano-Reggiano".

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3.4 Natural whey starter for Parmigiano Reggiano: culture independent approach

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Journal of Applied Microbiology, Submitted

Running headline: Parmigiano Reggiano whey starter

3.4.1 Abstract

Aims: Aim of this work was a deeper insight into the knowledge of microbial composition of Parmigiano Reggiano natural whey starters through different culture-independent methods.

Methods and Results: Eighteen Parmigiano Reggiano natural whey starters sampled from three different provinces of this cheese production area and the non acidified wheys from which they arose, have been studied by means of LH-PCR and FISH. A high microbial composition variability between different samples has been observed.

Conclusions: Revealing different images of the same community, LH-PCR and FISH gave a more accurate view of the little known Parmigiano Reggiano whey starter ecosystem.

Significance and Impact of the Study: New lights have been shed on Parmigiano Reggiano natural whey starters microbial composition, highlighting how culture-independent approach could be used and improved for studying this and other food ecosystems.

Key words: FISH, LH-PCR, microbial community, natural whey starter, Parmigiano Reggiano.

3.4.2 Introduction

Parmigiano Reggiano is an Italian protected designation of origin (PDO) cheese. It is a hard, cooked cheese made from raw partly skimmed cow's milk supplemented with natural whey starter (Neviani et al.

1998; Coppola et al. 2000). Natural whey starter is obtained from the previous day's cheese making whey incubated at a gradually decreasing temperature (Neviani and Carini 2004, Coppola et al. 1997). During cheese production, the composition of LAB (lactic acid bacteria) microflora undergoes several changes, according to modifications of environmental conditions, which frequently provide cellular stresses, such as heat shock, adverse pH, as well as reduction of oxidation-reduction potential, water activity, and nutrient content (Di Cagno et al., 2006). Thermophilic acidifying starters grow during the first hours of hard cooked cheese making, determining the key acidification step, drawing enzymatic potential and the environment that will prevail for microbial growth and activity throughout the ripening period (Charlet et al. 2008). Technological performances of these complex consortia of microorganisms are of great importance for the quality of an appreciated cheese such as Parmigiano Reggiano. The study of the dynamics of such populations have often been hampered by culturing techniques limitations, such as failure in detecting viable non cultivable bacterial species and selection given by chosen growing parameters (Fleet 1999, Giraffa and Neviani 2001). To overcome these drawbacks, different non cultivable methods have been developed and applied to whey starter or other food matrices, among which LH-PCR (length heterogeneity polymerase chain reaction) (Lazzi et al., 2004, Santarelli et al., 2008, Gatti et al., 2008) and FISH (fluorescent in situ hybridization) (Ercolini et al., 2003 a,b, Fornasari et al., 2008). To date, few studies on natural whey starters for Parmigiano Reggiano have been done (Cocconcelli et al., 1997; Coppola et al., 2000; Gatti et al., 2003; Gatti et al., 2008). Through both culture dependent and culture independent techniques, those authors, observed that the microflora of natural whey starters for Parmigiano Reggiano was constituted principally by *Lactobacillus helveticus*. With the awareness

of culture based methods limits in detecting microorganisms in complex ecosystems such as fermented foods (Jany et al. 2008), a deeper insight into the knowledge of microbial composition of natural whey starters for Parmigiano Reggiano was aimed in this study. Using a polyphasic approach, combining different culture-independent methods such as FISH and LH-PCR, aim of this work was to investigate the microbial population in eighteen Parmigiano Reggiano natural whey starters sampled from three different provinces of this cheese production area. Further, the microbial composition of the non acidified wheys from which the whey starters arose, was considered.

3.4.5 Materials and methods

Whey starter samples

Eighteen whey starters for Parmigiano Reggiano were sampled from nine different dairies located in the provinces of Parma (A, B, C), Modena (D, E, F) and Reggio Emilia (G, H, I), belonging to the area of production of the Parmigiano Reggiano PDO cheese. Whey cultures (named "a" as "acidified") were collected just before addition to vat milk. The eighteen non acidified wheys (named "na" as "non acidified") from which whey starters arose from, were collected from the vat ten minutes after the end of the cooking process. From each dairy, samples were collected for two consecutive days of cheesemaking (e.g.: A_{na1}, A_{a1}, A_{na2}, A_{a2}). Samples were cooled at 4-6°C at the dairy plant, shipped under refrigerated transport to the laboratory and analysed.

Microbial count

Natural whey starters and non acidified whey samples were serially diluted 10 folds in 0.05 mmol l^{-1} sodium citrate (Sigma, Italy) buffer pH 7.5. In order to recover the cultivable bacterial population, to facilitate the growth of the strains arising from natural whey starter, Whey agar

medium (WAM) incubated under anaerobic conditions at 42°C for 48h was used (Gatti et al 2003). Plate counts were carried out in duplicate. The enumeration of the total bacteria cells by the DNA intercalating agents DAPI (4,6-diamidine-2phenylindole 2 HCl) (Sigma-Aldrich, Milano, Italy) was performed using a procedure from Mesa et al. (2003). A stock solution of DAPI was prepared by dissolving 1 mg of DAPI in 10 ml of ultrapure water. The stain at a final concentration 10µ g/ml was added to 1 ml of second-diluted natural whey starter previously washed twice with sterilized distilled water and then incubated for 30 min at room temperature in the dark. After the incubation, the samples were filtered on a black polycarbonate membrane (Millipore corp., Billerica, MA, USA); the membrane was air-dried and mounted on glass slide in Citifluor solution (Citifluor Ltd, London, UK). The bacteria on DAPI-stained membranes were enumerated by counting the total number of blue fluorescing bacteria. The number of bacteria was estimated from counts of 20 microscopic fields (at X 1000) and calculated as follows:

$$\mathbf{N} = \frac{C \times A}{a \times V} \times D$$

where N is the number of cells ml^{-1}; C is the number of cells per observation field; A is the filtration area (mm^2); a is the observation field area (mm^2); V is the volume of sample filtered (ml); and D is the dilution factor.

FISH

Whey samples were washed twice in PBS and pellets were resuspended in 300 µl of PBS. Cells were fixed according to Amann et al. (1990) adding freshly prepared cold paraformaldehyde 4% (Sigma-Aldrich, Milano, Italy), 1:3 (v:v), and storing for 1h at 4°C. After washing, pellets were resuspended in 50% (v/v) ethanol/PBS and stored at -20°C until

further FISH analysis. About 20 µl of fixed cell suspension were spotted on poly-L-lysine coated slides and let dry at 46°C for 10 min in a oven. Spots were dehydrated in ethanol series by covering them with about 50 µl of 50, 80 and 100% ethanol solutions for 3 min each and air dried. Specimens were treated by covering the spots with 10 µl of proteinase K (10 mg ml -1) for 10 min at 37°C, to allow permeabilization of Gram positive cells. Lbh1-FITC labelled probe (5'-ACT TAC GTA CAT CCA CAG 3'), specific for *Lact. helveticus* and St4-Cy3 labelled probe (5'-TTA TCC CCC GCT ACA AGG 3') specific for *Strep. thermophilus* (Taillez 1997), have been used. Both probes were synthesized and labelled by MWG (Germany) After addition of 10 µl of the hybridization buffer (0.9 mol l^{-1} NaCl, 0.01% SDS, 20 mmol l^{-1} Tris-HCl pH 7.2, 45% formamide) containing 10 ng of Lbh1 probe and 10 ng of St4 probe, slides were incubated in a dark humid chamber at 45°C for 4 hours. Unbound oligonucleodtides were then removed by incubating slides in pre-warmed washing buffer (20 mmol l^{-1} Tris-HCl pH 7.2, 0.01% SDS, 40 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA) at 46° C for 15 min and by rinsing with water.

Slides embedded in mounting oil were evaluated with an Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp. Nikon filter set B2A FITC was used for Lbh1 FITC labelled probe (excitation wavelength, 450-490 nm; emission wavelength, 500–520 nm). Nikon filter set G-2E/Cwas used for St4 Cy3 labelled probe (excitation wavelength, 540/25 nm; emission wavelength, 605/55 nm). Pictures of each field were taken then superimposed through the Nis Elements software (version 2.10 Nikon).

DNA extraction

DNA extraction for each sample was done by means of InCura DNA extraction kit (InCura srl, Cremona, Italy) according to manufacturer instruction. DNA was spectrophotometrically (Jasco V-530, Japan) quantified by measuring absorbance at 260 nm, diluted up to 20 ng μl^{-1} and stored at -20°C until use.

LH-PCR

LH-PCR to analyse the Domain A of the variable region of the 16S rRNA gene was performed as previously described by Lazzi et al. (2004). The primer pair 63F (5'-AGGCCTAACACATGCAAGTC-3') 5' end labeled with 6-carboxy-fluorescein (6-FAM) and 355R (5'-GCTGCCTCCCGTAGGAGT-3') were used for the analysis. Reaction and amplification conditions proposed by Lazzi et al. (2004) were slightly modified according to Gatti et al. (2008). 0.5 U of Platinum Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) were used in 20 µl of reaction mixture. Initial denaturation at 94°C for 2 min was followed by 25 cycles consisting of denaturation at 95°C for 45 s, annealing at 49°C for 45 s, and extension at 72°C for 2 min. A final extension step of 72°C for 7 min was done. LH-PCR products were stored at -20°C in the dark until use (usually less than 1 week). For fragment analysis, 1 µl volumes of LH-PCR amplicons were mixed with 12 µl of deionized formamide (Applied Biosystems, Foster City, CA, USA) and with 1 µl of internal size standard (GS500 LIZ®, Applied Biosystems, Foster City, CA, USA) and then denatured at 90°C for 2 min followed by immediate chilling on ice. Capillary electrophoresis on the ABI Prism 310 genetic optimized polymer 4 (POP-4™), 15 s injection time, 15 kV injection voltage, 35 min electrophoresis at 15 kV and 60°C capillary temperature. The peaks of the electropherogram

profiles, corresponding to amplicons of different length, were attributed to bacterial species according to an LH-PCR database obtained in a previous study (Gatti et al. 2008). Amplicons sizes were determined with GeneMapper v4.0 software (Applied Biosystems, Foster City, CA, USA). LH-PCR profiles were analysed by reference to the internal size standard using the local Southern size calling method and no-smoothing option, and a threshold of 50 fluorescent units was used.

3.4.6 Results

Eighteen natural whey starters and the eighteen non acidified wheys from which they arose, sampled from nine different dairies located in different zones of Parmigiano Reggiano production, were investigated. Plate counts on WAM showed a very similar trend of cultivability among all whey starter samples. Lactic acid bacteria able to grow in WAM ranged between 8.11 and 8.79 log CFU ml $^{-1}$, with a variability lower than 10% (Table 1).

Table 1 Mean log count* of total microbial population and cultivable in WAM microbial population of 18 natural whey starters

	Microbial log counts and standard deviation (SD)			
	Total expressed as cells number ml^{-1}		cultivable in WAM expressed as mean cfu ml^{-1}	
Samples	Mean	SD	Mean	SD
A_{a1}	9,15	0,12	8,03	0,51
A_{a2}	9,20	0,15	8,09	0,71
B_{a1}	9,26	0,18	8,36	0,78
B_{a2}	9,58	0,32	8,40	0,55
C_{a1}	9,89	0,16	8,64	0,88
C_{a2}	9,65	0,28	8,56	0,81

D_{a1}	9,51	0,43	8,34	0,66
D_{a2}	9,16	0,11	8,26	0,45
E_{a1}	9,46	0,15	8,43	0,78
E_{a2}	9,63	0,33	8,48	0,74
F_{a1}	9,94	0,14	8,79	0,89
F_{a2}	9,80	0,10	8,77	0,81
G_{a1}	9,52	0,28	8,11	0,63
G_{a2}	9,48	0,22	8,39	0,52
H_{a1}	9,47	0,17	8,73	0,59
H_{a2}	9,64	0,18	8,42	0,63
I_{a1}	9,61	0,25	8,29	0,75
I_{a2}	9,65	0,25	8,31	0,46

A culture-independent approach was used then, in order to find confirmatory, complementary or alternative results. Total cell count in natural whey starter samples ranged between 9.15 and 9. 94 log cell ml $^{-1}$ with a variability lower than 5% (Table 1) . Whey starters and non acidified wheys species composition were investigated by means of LH-PCR. Three representative LH-PCR profiles of whey starters are shown in Fig. 1.

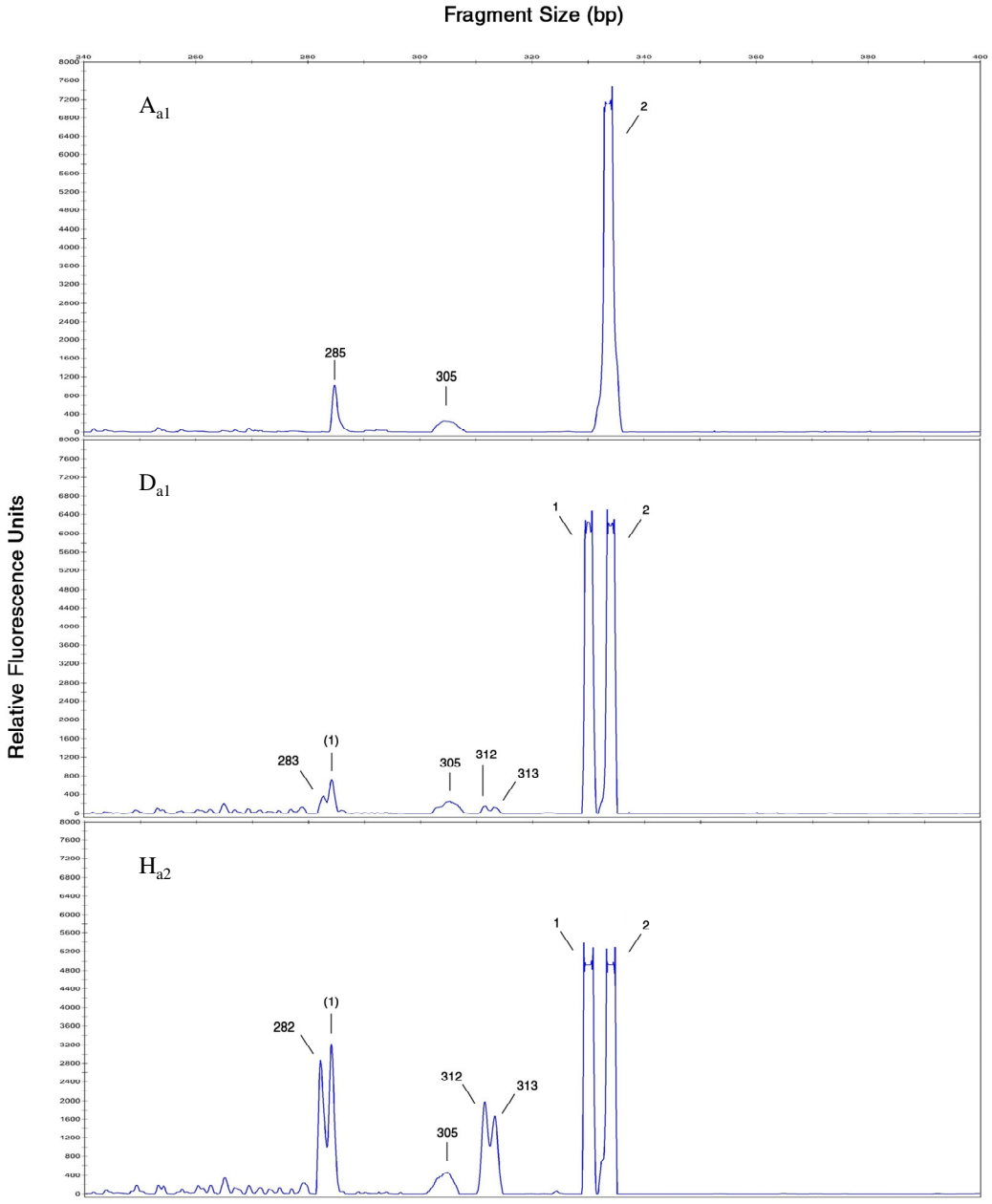


Fig. 1 Length heterogeneity LH-PCR electropherograms of three whey starter samples representative of different cases occurring: *Lact. helveticus* as dominant species (a), comparable percentages of *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* (b), *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* percentages comparable also to the percentage of other species (c). The *x* axis shows peaks size in base pairs, and the *y* axis shows peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to LH-PCR published database as follows: 1 *Lact. delbrueckii* subsp. *lactis*; (1) Secondary peak of *Lact. delbrueckii* subsp. *lactis*; 2, *Lact. helveticus*; 282, 283, 285, 305, 312, 313 are non attributed peaks.

The different fragment sizes in the LH-PCR profiles were attributed to bacterial species according to published LH-PCR database (Lazzi et al., 2004, Fornasari et al., 2006, Gatti et al., 2008). Since the areas under the peaks shown in the electropherograms are rough measure of the proportions of the species, their relative estimation was also possible (Suzuki et al., 1998). For the majority of samples, some peaks were most frequently detected: 330 ± 1 bp (attributed to *Lactobacillus delbrueckii* subsp. *lactis* or subsp. *bulgaricus*), 334 ± 1 bp (*Lact. helveticus*), 319 ± 1 bp (*Strep. thermophilus*), 342 ± 1 bp and 345 ± 1 bp (attributed to *Lactobacillus fermentum*) and 305 ± 1 bp (non attributed). However no peak at 330 ± 1 bp (attributed to *Lact. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*) was detected in samples A_{na1}, A_{a1}, A_{na2} and A_{a2}. A peak attributable to *Strep. thermophilus* was revealed in the whey starters B_{a1}, C_{a1}, $G_{a1,}$ D_{a2}, E_{a2}, electropherograms. Two fragments of 342 ± 1 bp and 345 ± 1 bp revealed the presence of *Lact. fermentum* in the whey starters samples B_{a1} and B_{a2}, F_{a1}, F_{na2}, F_{a2} , G_{a1} G_{a2}, and I_{a2}. We noticed that for samples G_{na1}, $G_{na2,}$ H_{na1}, I_{na1} and I_{na2} the 330 ± 1 bp peak (attributed to *Lact. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*) fluorescence intensity was lower than in the deriving whey starters. H_{a1} and H_{a2} whey starters and H_{na1} and H_{na2} non acidified wheys profiles were characterized respectively by the presence of two peaks at 312 and 313 ± 1 bp and at 305 ± 1 bp, non attributed (data not shown). The composition of natural whey starters and non acidified wheys sampled in this study, has been investigated also by FISH. 23S rRNA Lbh1 probe specific for *Lact. helveticus* and 16S rRNA St4 probe specific for *Strep. thermophilus* have been used. Being respectively labelled with FITC (green) and Cy3 (red), the simultaneous visualization of both groups of hybridized cells was possible. For each sample, green hybridized rod-shaped cells (*Lact. helveticus*), red hybridized round-shaped cells (*Strep. thermophilus*) and

non hybridized rod-shaped cells were observed (Fig. 2). Non hybridized cells could be either Lactobacillaceae non *Lact. helveticus* or non viable (low RNA content) *Lact. helveticus*.

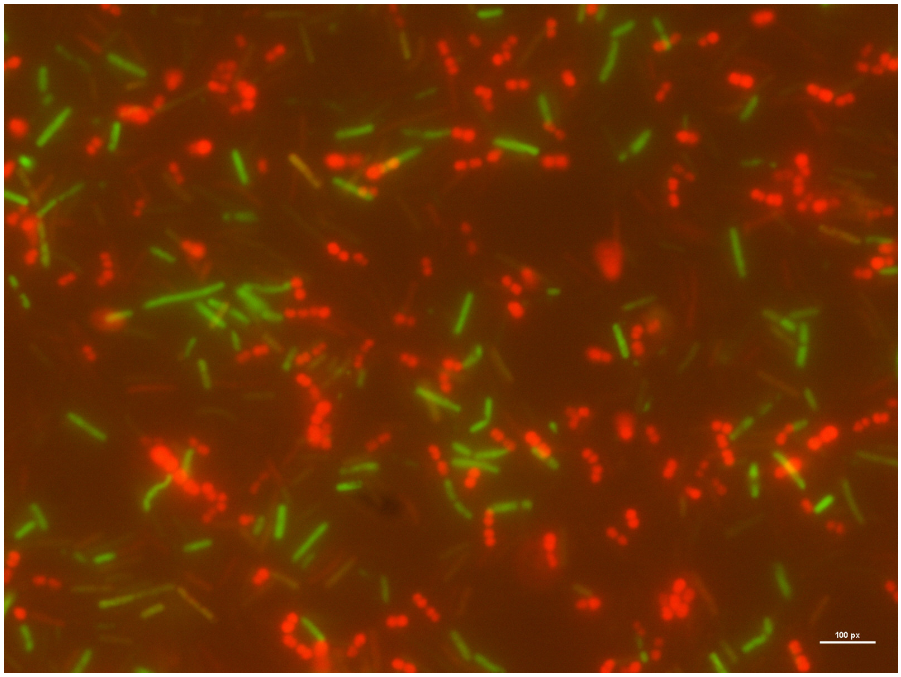
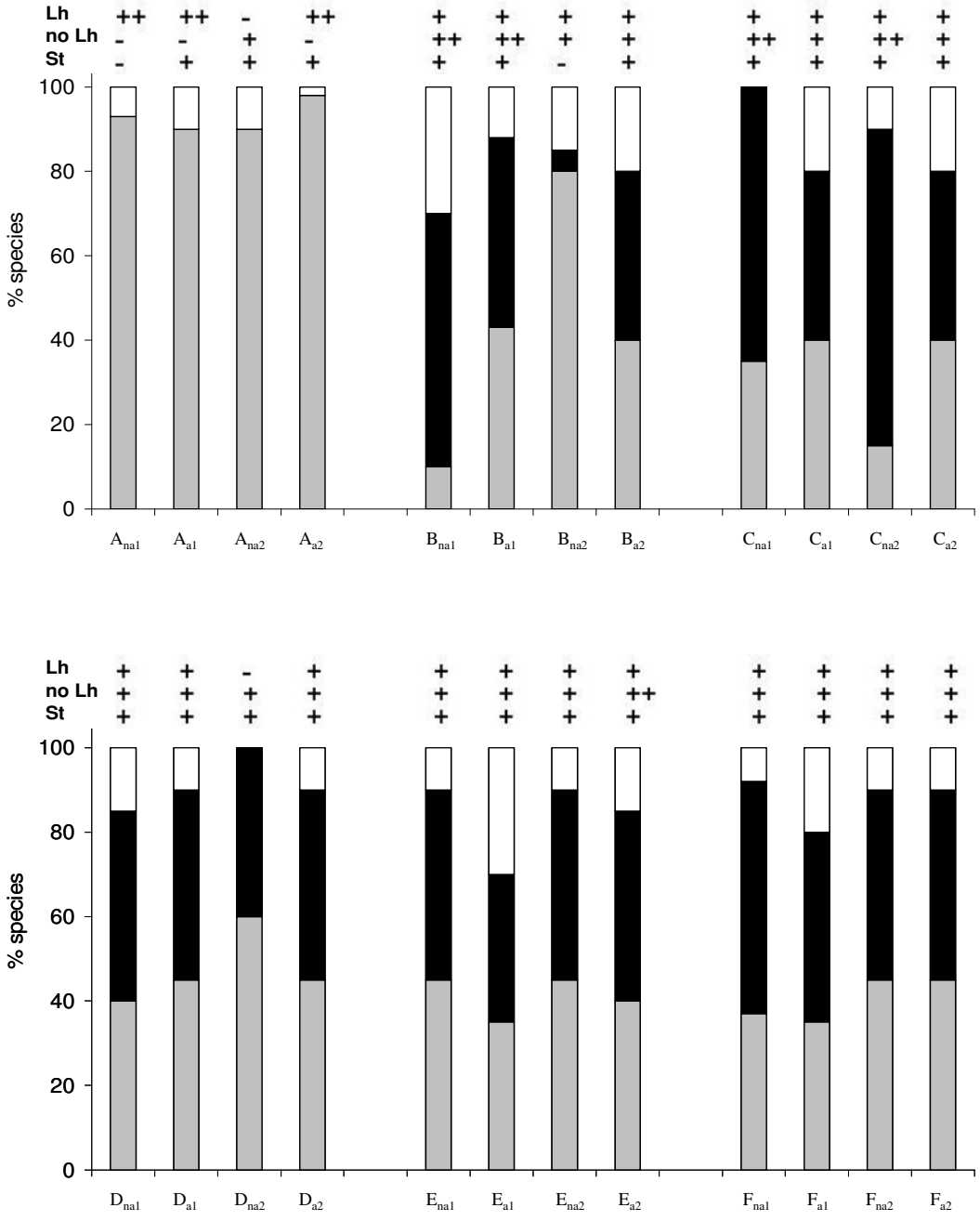


Fig. 2 FISH on a sample of Parmigiano Reggiano natural whey starter. Simultaneous use of probes St4 (red label) and Lbh1 (green label). Hybridized *Lact. helveticus* cells appear green, Hybridized *Strep. thermophilus* cells appear red. Non hybridized cells on the background could be either Lactobacillaceae non *Lact. helveticus* or non viable *Lact. helveticus*

The signal intensity of cells hybridized with oligonucleotide probes is in fact directly related to the cellular rRNA content (Bottari et al., 2006) which is a useful indicator of viability (Bentsink et al., 2002). Positivity to one or more of these conditions was indicated with "+" or "++" if representing the majority. A majority of *Lact. helveticus* hybridized cells was observed for A_{na1}, A_{a1}, A_{a2}, H_{a2} and I_{a1}. Non hybridized rod-shaped cells were found to be the major component for B_{na1}, B_{a1}, C_{na1}, C_{na2}, E_{a2}, G_{a1}, G_{na2}, G_{a2}, H_{na1}, H_{na2}, I_{na2} and I_{a2}. Hybridized cells of *Strep. thermophilus* have been observed for all the natural whey starter

samples, and for almost all the non acidified whey samples, except for A_{na1}, B_{na2}, G_{na2} and I_{na1} (Fig. 3).



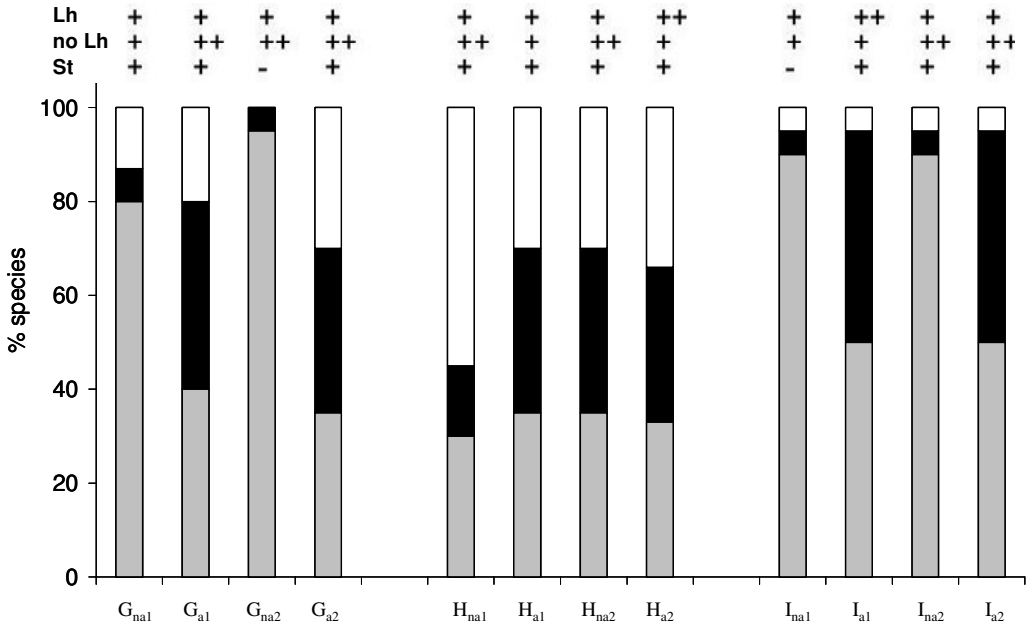


Fig. 3 LH-PCR (bars) and FISH (grid) results for whey starter samples ($samples_a$) and non acidified whey samples ($samples_{na}$). Bars- Percentage of *Lact. helveticus* species (grey), *Lact. delbrueckii* species (black) and other species (white) calculated on peaks relative abundances in LH-PCR profiles. Grid- Presence (+) or absence (-) of hybridized *Lact helveticus* cells (Lh), hybridized *Strep. thermophilus* cells (St) and non hybridized cells (no Lh) revealed by FISH. (++) indicates majority.

3.4.7 Discussion

A polyphasic approach, combining different culture-independent methods such as FISH and LH-PCR, was used to investigate the microbial population in eighteen Parmigiano Reggiano natural whey starters sampled from three different provinces of this cheese production area. In previous works, plate isolation revealed a simple composition of Parmigiano Reggiano whey starters microbiota, highlighting the prevalence of *Lact. helveticus* species and the presence of *Lact. delbrueckii* (Cocconcelli et al., 1997; Coppola et al., 2000). The always more used culture-independent methods for the analysis of microorganisms in food, have already shed light on the structure of microbial population of dairy environment (Andrighetto et al., 1998;

Fitzsimmons et al., 1999; Mannu et al., 2000; Berthier et al., 2001; Dasen et al., 2003). The use of LH-PCR and FISH, has been therefore chosen for a better knowledge of such a little known ecosystem as Parmigiano Reggiano whey starter, and performed together with traditional counting methods. Plate counting was performed on WAM which, as proven by Gatti et al (2003), gives better results in counting natural whey starter lactic acid bacteria, thank to its capability of reproducing the natural system of the whey. Despite the use of WAM, the number of CFU ml $^{-1}$ were even than 1 unit log lower than direct total count measured with DAPI (Tab. 1). These results confirm that cultivability is lower than the total number of cells present in a whey starter sample, as previously observed by Gatti et al. (2006) for Grana Padano natural whey starters. Moreover, the low variability revealed by traditional culture-dependent approach, could lead to the conclusion, at first, that all whey starter samples are very similar. However, possible biases could have been introduced by culturing, such as failure in detecting species which are not able to grow under selected experimental conditions and the actual microbial diversity could have been misinterpreted. In order to overcome this bias, and to gain a deeper knowledge of whey starter microbial composition, LH-PCR and FISH have been used. LH-PCR electropherograms of almost all samples, revealed the presence of most frequently detected peaks, attributable to *Lact. helveticus, Lact. delbrueckii* subsp. *lactis*, *Strep. thermophilus* and *Lact. fermentum.* According to published database (Lazzi et al., 2004, Fornasari et al., 2006, Gatti et al., 2008), 330 ± 1 bp peak could be attributable also to *Enterococcus faecium* or *Enterococcus faecalis*. However, in whey samples microscopically observed after FISH analysis, all present round-shaped cells were hybridized by *Strep. thermophilus*-specific probe St4. Excluding therefore the presence of Enterococcaceae, peak at 330 ± 1 bp has

been attributed to *Lact delbrueckii* species. Our results are in agreement with Lazzi et al. (2004), Fornasari et al. (2006), Santarelli et al. (2008) and Rossetti et al. (2008), who observed that the microbial composition of natural whey starters for another Italian hard cooked cheese such as Grana Padano shows a constant presence of dominant species corresponding to *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* and minor species corresponding to *Strep. thermophilus* and *Lact. fermentum*. Nevertheless, our results revealed a higher variability in the composition of different whey starters for Parmigiano Reggiano in comparison with the only natural whey starter for Parmigiano Reggiano to date analysed by LH-PCR (Gatti et al., 2008). Through culture-dependent approach, previous works on Parmigiano Reggiano natural whey cultures, ascribed predominant whey microflora to *Lact. helveticus* (Cocconcelli et al., 1997; Coppola et al., 2000 Gatti et al., 2003). In our study, LH-PCR results, differently from plate counting, highlighted a various microbial composition among whey starters. In fact, only two samples (A_{a1}, A_{a2}) were characterized by *Lact. helveticus* as dominant species (Fig. 3), while the majority of whey starter samples, were found to have comparable percentages of *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* (B_{a1}, B_{a2}, C_{a1}, C_{a2}, D_{a1}, D_{a2}, E_{a2}, F_{a1}, F_{a2}, G_{a1}, I_{a1}, I_{a2}). In few whey starters (E_{a1}, G_{a2}, H_{a1}, H_{a2}), *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* percentages were also comparable to the percentage of other species, represented by non attributed and attributed peaks among which *Strep. thermophilus* (Fig. 3). We noticed that whey starter samples with *Lact. helveticus* as dominant species (A_{a1}, A_{a2}), arose from non acidified wheys where *Lact. helveticus* was the dominant species (A_{na1}, A_{na2}). Otherwise, other non acidified wheys with a higher percentage of *Lact. helveticus* (B_{na2}, D_{na2}, G_{na1}, G_{na2}) gave rise to whey starters where *Lact. helveticus* was not the dominant species (B_{a2}, D_{a2}, G_{a1}, G_{a2}) (Fig. 3). This variability could be

related to different incubation conditions used by each dairy for the production of natural whey culture. The microbial composition and diversity of whey starters is, in fact, modulated by several factors, among which high thermophilic condition and deep acidification rate, responsible for a technological selection of microorganisms (Neviani et al., 1995, Giraffa et al., 1998, Fortina et al., 1998, Giraffa et al 2004). Any small variation of temperature gradient decreasing or final acidity level, could therefore justify the variability observed. These differences could be minimized by traditional cultural approach through which the cultivation of species more resistant to extreme whey environment conditions, such as *Lact. helveticus* and *Lact. delbrueckii,* is favourite. Otherwise, a culture-independent approach could be useful to study the less known component of whey microbial population and eventually its technological role. Whey starters and non acidified wheys have been then analysed by FISH. Being respectively the dominant species and one of the most frequently detected minority species in whey starters for hard cooked cheeses (Cocconcelli et al., 1997; Coppola et al., 2000 Gatti et al., 2003, Rossetti et al., 2008), *Lact. helveticus* and *Strep. thermophilus* have been chosen as targets for FISH experiments. Considering natural whey starter samples, FISH results were in good agreement with LH-PCR ones. However, a higher number of samples containing *Strep. thermophilus* was revealed by FISH. Both techniques have a limit of detection around 10^4 - 10^5 cell ml^{-1} (Lazzi et al., 2004, Fornasari et al., 2008), but with a very high percentage of *Lact. helveticus* or other species in the samples, *Strep. thermophilus* could have gone undetected by LH-PCR. This could explain the better effectiveness of FISH in detecting *Strep. thermophilus*. Discordance between FISH and LH-PCR results was observed for some non acidified whey samples (A_{na1}, A_{na2}, D_{na2}). Targeting respectively RNA and DNA, FISH and LH-PCR are able to

detect cells in a good physiological state the former and both living and dead cells the latter. Cooking could affect integrity and thus lower RNA content of non acidified whey microbial cells, making some of them non detectable by FISH. Otherwise, DNA of both live and dead or damaged cells can be estimated by LH-PCR (Gatti et al. 2008); slight differences observed with these two methods in non acidified whey samples could be therefore explained.

An overall picture of the microflora of eighteen Parmigiano Reggiano cheese whey starters was determined by LH-PCR analysis. This culture-independent approach shed light on a great variability among the studied whey starter samples differently from what observed with traditional culture based techniques. A further knowledge on microbial composition of whey starter samples has been added with FISH analysis. These methods revealed different images of the same community, therefore a polyphasic approach, combining LH-PCR and FISH was worthwhile to obtain a more accurate view of the structure of Parmigiano Reggiano whey starters microbial community. Nevertheless, the frequent presence of several non attributed peaks shown by LH-PCR in whey starter and non acidified whey electropherograms, draws attention on the fact that culture independent methods need to be improved to reveal as accurately as possible the actual microbial composition of wheys and other food ecosystems. Further efforts might be therefore devoted in the future to address LH-PCR, as well as other culture-independent techniques, to a deeper knowledge of what these techniques still do not exhaustively describe.

3.4.8 References

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4 General conclusions

Foods are teeming with microorganism that may be innocuous, pathogenic threats, spoilage agents, or beneficial microorganisms driving fermentations or acting as biocontrol agents.

Great efforts have been made to date in order to evaluate the presence and the activity of the whole food microbiota. However, traditional methods for studying microbial population, like plating on selective media, commonly detect the most frequently occurring organisms that can grow to a detectable level by forming colonies on selective media. Thus, trying to shed light on what traditional culture-dependent methods left hanging, aim of this PhD thesis, was to find new ways to study microorganisms in food. Considered techniques were found to be unsuitable for the direct research of minority populations such as pathogens. The actual applicability of those methods to food ecosystems was then challenging evaluated in studying three different fermented food matrices. Fluorescent staining, FISH (fluorescence in situ hybridization) and LH-PCR have been used to gain a deeper knowledge of microbial composition and dynamics of different food matrices, such as artisanal beer, Parmigiano Reggiano natural whey starter and Parmigiano Reggiano cheese. FISH technique has been found to have the potential of studying the spatial distribution of microbial population in situ in foods, allowing for the location of specific groups of bacteria within the food matrix, and the investigation of relationships existing within specific groups of bacteria. A FISH protocol for the analysis of food samples has been developed. Based on fluorescence as well as FISH, viability measurement has been held to monitor yeast activity through pitching and reuse cycles in artisanal brewing. Results suggested that periodical evaluation of this parameter, could be the starting point for an effecting yeast management program, in order to maintain or even improve the quality

properties of the final beer. Afterward, length heterogeneity PCR (LH-PCR) technique was applied to monitor the microbial succession in a complex fermented ecosystem as Parmigiano Reggiano cheese. The use of this culture independent method overcame traditional agar plate and culture dependent method limitations. The modality of LH-PCR samples preparation allowed to evaluate both the entire and lysed cells evolution during cheesemaking and ripening. Thermophilic microflora of natural whey starter, added to drive the cheese fermentative process, was the dominant population until the second months of ripening. In subsequent months, until the sixth, a trend reversal was observed in LH-PCR data, confirming that environmental conditions in this phase, favour mesophilic microflora development since their overcoming. This approach has never been used to study a ripened cheese where viable, not viable and lysed microbial cells coexist. In the wake of results obtained for Parmigiano Reggiano cheese, the potential of LH-PCR has been exploited for studying natural whey starter for Parmigiano Reggiano. LH-PCR has been combined with FISH, for a better knowledge of such a little known ecosystem. Results highlighted a various microbial composition among different whey starter samples, differently from what observed with traditional culture based techniques. Samples characterized by *Lact. helveticus* as dominant species, samples with comparable percentages of *L. helveticus* and *Lact. delbrueckii*, and samples with *Lact. helveticus* and *Lact. delbrueckii* percentages also comparable to the percentage of other species, among which *Strep. thermophilus,* have been observed.

Results obtained during this PhD thesis, draw attention on culture-independent methods potential for studying microorganisms in food. These methods can overcome biases introduced by culture-based techniques, allowing to consider the association microorganism-food from another perspective. Nevertheless, their frequent failure in

indentifying species obtained using culture-dependent methods, suggests the complementary use of both the approaches. Thus, culture-independent methods still need to be improved to reveal as accurately as possible the actual microbial food ecosystems. Further efforts might be therefore devoted in the future to address these techniques to a deeper knowledge of what they still don't exhaustively describe.

5. CURRICULUM VITAE

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Studies

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