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Novel extraction strategy of ribosomal RNA and genomic DNA from cheese for PCR-based investigations

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Abstract

Cheese microorganisms, such as bacteria and fungi, constitute a complex ecosystem that plays a central role in cheeses ripening. The molecular study of cheese microbial diversity and activity is essential but the extraction of high quality nucleic acid may be problematic: the cheese samples are characterised by a strong buffering capacity which negatively influenced the yield of the extracted rRNA. The objective of this study is to develop an effective method for the direct and simultaneous isolation of yeast and bacterial ribosomal RNA and genomic DNA from the same cheese samples. DNA isolation was based on a protocol used for nucleic acids isolation from anaerobic digestor, without preliminary washing step with the combined use of the action of chaotropic agent (acid guanidinium thiocyanate), detergents (SDS, *N*-lauroylsarcosine), chelating agent (EDTA) and a mechanical method (bead beating system). The DNA purification was carried out by two washing steps of phenol–chloroform. RNA was isolated successfully after the second acid extraction step by recovering it from the phenolic phase of the first acid extraction. The novel method yielded pure preparation of undegraded RNA accessible for reverse transcription–PCR. The extraction protocol of genomic DNA and rRNA was applicable to complex ecosystem of different cheese matrices.

Keywords: Reverse transcriptase PCR; Cheese; rRNA and DNA extraction; Microbial diversity; Microbial activity

1. Introduction

Soft smear-ripened cheeses are characterised by a complex microbial flora on their surface. This flora has a strong impact on the appearance, flavour and texture of the cheese and reduces the ripening time of soft smear varieties to several weeks. Traditional microbiological methods, based on the cultivation of microorganisms prior to characterization by physiological and biochemical tests or by molecular methods, can give significant insight into specific isolates, microbial populations and into the evolution and the nature of the microbial groups during cheese ripening. That is how, *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Kluyveromyces lactis* or *Kluyveromyces marxianus* and *Yarrowia lipolytica* became known to be the most frequently isolated species from smear cheeses (Seiler and Busse, 1990; Eliskases-Lechner and Ginzinger, 1995a,b; Prillinger et al., 1999). The cheese surface colonization by the salt-tolerant and acid-sensitive bacteria such as Gram-negative bacteria, coryneform bacteria and staphylococci is promoted by a yeast effect of increasing the pH (Reps, 1993; Eliskases-Lechner and Ginzinger, 1995a,b; Valdes-Stauber et al., 1997). *Staphylococcus equorum*, *Staphylococcus vitulinus* and *Staphylococcus xylosus* are the staphylococci species most often identified in traditional cheeses manufactured with raw milk (Irlinger et al., 1997). But many investigations have also shown that coryneform bacteria, including a large number of species, particularly *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Corynebacterium*, and *Microbacterium*, are important (Seiler, 1986; Eliskases-Lechner and Ginzinger, 1995a,b; Bockelmann et al., 1997; Valdes-Stauber et al., 1997; Carnio et al., 1999; Hoppe-Seyler et al., 2000; Bockelmann and Hoppe-Seiler, 2001; Brennan et al., 2002). However, cultivation methods can over- or underestimate the microbial diversity, as media may be too selective (Hugenholtz et al., 1998). It has been reported by Amann et

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al. (1995) that only a small fraction of microorganisms is analysed by conventional methods and often, the isolated strains do not seem to represent the real spectrum of microorganisms and their active genes in the habitat of choice. In addition, methods to understand the link between structural diversity and functioning of complex ecosystem need to be developed so that the questions of how diversity influences functions can be addressed. Novel molecular approaches, especially those based on the use of rRNA and DNA, have provided the opportunity to analyze complex communities on the basis of sequence diversity: denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Coppola et al., 2001; Randazzo et al., 2002) and the related techniques, temperature gradient gel electrophoresis (TGGE) (Cocolin et al., 2000a,b), temporal temperature gel electrophoresis (TTGE) (Ogier et al., 2002), terminal-restriction fragment length polymorphism (T-RFLP) (Osborn et al., 2000), and single-strand conformation polymorphism (SSCP), have been applied in microbial ecology to resolve PCR-amplified regions of 16S rRNA genes or rRNA based solely on differences in nucleotide sequences. These direct molecular techniques have proven to be valuable approaches to compare structure of complex microbial communities and to monitor their dynamics in relation to environmental factors in several food ecosystem (Ampe et al., 1999; Cocolin et al., 2000a,b, 2001; Coppola et al., 2001; Duthoit et al., 2003; Giraffa, 2004; Feurer et al., 2004a,b). The PCR–SSCP method has been especially applied to study microbial community in water (Lee et al., 1996), in compost (Peters et al., 2000), in maize rhizospheres (Schmalenberger and Tebbe, 2003), and in anaerobic digestors (Delbès et al., 2000, 2001; Dabert et al., 2001; Leclerc et al., 2001). In clinical microbiology, it has also been used to rapidly differentiate bacteria from blood culture (Turenne et al., 2000) or bacterial cell lysates (Widjojoatmodjo et al., 1994) and to detect single base substitution (Sheffield et al., 1993). In order to notify a change in the bacterial community structure and activity, during cheese manufacture, some authors have extracted reverse transcription PCR-quality DNA and rRNA from milk and cheese for DGGE or PCR–SSCP analysis (Randazzo et al., 2002). Comparison of the DNA- and RNA-derived DGGE or PCR–SSCP profiles revealed the metabolically active members (Randazzo et al., 2002) but also showed that cheese is a medium rich in nuclease and inhibitory components of PCR (Rossen et al., 1992). Actually, it has been shown that the isolation of reverse transcription quality RNA from the cheese seems to be a sensitive procedure (Randazzo et al., 2002).

Only few communities composed of prokaryotes and eukaryotes have been studied by using a simultaneous RNA and DNA extraction method (Majumdar et al., 1991; Peters et al., 2000). No one, as far as we know, has carried out a study on food complex microbial consortium such as cheese. Indeed, studies of cheese microbial diversity were based only on the nucleic acids extraction either from prokaryote (Randazzo et al., 2002) or from eukaryote (Cappa and Cocconcelli, 2001) communities.

The present study describes a simple and fast method to isolate total RT–PCR quality RNA and DNA, and a cheese medium composed of a mixed consortium of yeast and bacteria is used as a model. This procedure leads to using a RT–PCR–SSCP analysis, enabling to detect specifically various viable and active bacteria and yeasts in cheese which are easily transposable to other complex ecosystems. The bias inherent in culture-independent approaches, especially the nucleic acids isolation, for evaluating microbial community in food ecosystem is discussed.

2. Materials and methods

2.1. Samples

2.1.1. Cheese experimental curd samples

A mixture composed of one *Staphylococcus*, four coryneform bacteria and two yeasts was inoculated on a cheese experimental curd similar to the Livarot cheesemaking. This curd was prepared on a pilot scale under aseptic conditions in a sterilized chamber and was ground with saline water (1.7% w/w NaCl). This experimental medium was been inoculated with 10^4 CFU/g of yeast and 10^6 CFU/g of bacteria. Experimental cheese curds were incubated at 14 °C in a ripening chamber with control of environmental parameters (temperature and relative humidity). Analyses were conducted at 0, 18 and 32 days.

2.1.2. Livarot cheese sample

Livarot is a smear soft cheese made with raw milk in Normandy. The Livarot cheese sample was made by a manufacturer that produces RDO (Registered Designation of Origin) Livarot cheese. The rind (2 mm thick) was sampled only at a ripening time corresponding to an optimal consumption date. The smear cheese microflora was not known.

2.1.3. Pure cultures: bacterial and yeast strains

All microorganisms used in this study were provided by the LGMPA laboratory (Grignon, France). They were isolated from the surface microbiota of different smear cheeses. They included five bacteria (*Staphylococcus equorum* (H4), *Brevibacterium linens* (127), *Brachybacterium alimentarium* (H9), *Arthrobacter* sp. (P7) and *Corynebacterium casei* (4_8)) and two yeasts (*Debaryomyces hansenii* (47_3) and *Geotrichum candidum* (124)). Bacteria were grown in 25 mL of broth heart infusion (Biokar Diagnostics, Beauvais, France) for 48 h at 25 °C and shaken at 150 rpm. Yeasts were grown in 25 mL of potato dextrose broth (Difco, Sparks, USA) for 48 h at 25 °C and shaken at 150 rpm.

2.2. Extraction and purification of total DNA and RNA from cheese experimental curds and Livarot cheese

All glassware were sterilized by heating at 200 °C overnight. All solutions were prepared with diethylpyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO, USA) treated water and autoclaved at 121 °C for 20 min. Plastic