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Application of reverse transcriptase PCR-based T-RFLP to perform semi-quantitative analysis of metabolically active bacteria in dairy fermentations

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Abstract

A method consisting of reverse transcriptase (RT)-PCR amplification of 16S rRNA from the total microbial community, coupled with T-RFLP, was optimized for semi-quantitative characterization of the metabolically active population in defined strain cultures of *Lactococcus lactis* ssp. *lactis* and *Leuconostoc citreum*, two mesophilic lactic acid bacteria (LAB) species routinely used in cheese manufacture. The set of PCR primers selected efficiently amplified the 16S rRNA from both bacterial species. The digestion of the PCR products with *Dde*I yielded different terminal restriction fragments (T-RFs) for each species. Nevertheless, additional T-RFs due to formation of chimeric molecules and pseudo-T-RFs derived from partly single-stranded 16S rRNA amplicons were observed in both species, although in minor amounts. Twenty PCR cycles were determined as the optimum to minimize the presence of artifactual fragments and to avoid underestimation of populations due to the saturation effect on DNA quantification caused by a PCR product excess. T-RFLP analysis showed a good repeatability when applied to mixed dairy cultures. Dynamics of two defined mixed starters consisting of a *L. lactis* ssp. *lactis* strain and a *L. citreum* strain were studied by this method and results compared to those obtained by a culture-dependent technique. The data indicated the suitability of T-RFLP to perform semi-quantitative analyses of microbial populations. Some slight differences could be explained by the presence of metabolically active cells that could not be detected by colony counting. RT-PCR-based T-RFLP can be an alternative to classical methods in order to study dynamics of metabolically active populations in relatively simple microbial ecosystems, such as defined dairy starter cultures.
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1. Introduction

Starter cultures used in manufacturing of several cheeses are usually composed of acid-forming lactococci, together with citrate-utilizing strains usually belonging to *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp. Such cultures are of great importance for the development of the organoleptic characteristics (Cárcoba et al., 2000). The use of defined-strain starters has been progressively adopted by cheese manufacturers as an attempt to solve the problems associated to uncontrolled natural fermentation. This has resulted in a positive effect on cheese quality. Since strain balance throughout fermentation is a key issue in the process, it is necessary to monitor any temporal effects on bacterial community structure that could alter the correct development of the desired characteristics of cheese, such as acidification rate or flavour production (Giraffa, 2004).

Cultivation has been for decades the main approach for evaluating the diversity and dynamics of microbial communities in foods. Cultured-based methods have had long-term acceptance and general success but these techniques are time and labour consuming and in some cases do not necessarily provide reliable information on the composition of microbial communities. The presence of unknown and non-cultivable species, as well as the effect of sample blending and dilution steps on the viability of the microorganisms, can lead to a distorted view of the real ecological composition of the product (Fleet, 1999). Moreover, under adverse conditions such as nutrient depletion, low temperature or other stresses, some microorganisms can enter in a viable but non-cultivable (VNC) state. VNC microorganisms are still metabolically active but do not form colonies on media that normally support their growth and, therefore, they cannot be detected by classical methods. To overcome these problems, culture-independent methods such as denaturing/thermal gradient gel electrophoresis (DGGE/TGGE), single strand conformation polymorphism (SSCP), fluorescence in situ hybridization (FISH) or PCR-based techniques such as length heterogeneity-PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) have been developed in the last years (Giraffa and Neviani, 2001). T-RFLP is a method that analyses variation among 16S rRNA genes from different bacteria and gives information of microbial community structure (Osborn et al., 2000). It is based on the restriction endonuclease digestion of fluorescent end-labelled PCR products. The individual terminal restriction fragments (T-RFs) are separated by gel electrophoresis and the fluorescence signal intensities are quantified. Depending on the species composition of the microbial community, distinct profiles (T-RF patterns) are obtained as each fragment represents each species present. A relative quantitative distribution can be obtained, since the fluorescence intensity of each peak is proportional to the amount of genomic DNA present for each species in the mixture. Nevertheless, PCR bias could negatively affect the quantification of the real composition of the microbial community (Suzuki and Giovannoni, 1996; Suzuki et al., 1998).

A disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms, since DNA from lysed cells can persist for long times in natural environments (Bentsink et al., 2002; Wolffs et al., 2005). Therefore, DNA-based PCR cannot differentiate among viable, VNC, and dead cells, which limits its use for monitoring purposes (Sheridan et al., 1998). RNA is degraded more rapidly than DNA upon cell death (Van der Vliet et al., 1994) and, therefore, its detection by reverse transcriptase PCR (RT-PCR) might be a good indicator of viability, metabolic activity or integrity of cells (Bentsink et al., 2002).

The aim of this work was to assay the ability of the T-RFLP analysis coupled with RT-PCR for monitoring the population dynamics of the metabolically active fraction of well-defined microbial communities such as dairy defined-strain starters. In this regard, the evolution of the two components of two defined-strain starters throughout the fermentation process was studied. The method was optimized in order to reduce PCR bias and its repeatability was tested.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study included two *Leuconostoc citreum* strains (*IPLA621* and *IPLA1687*) and a *L. lactis* ssp. *lactis* strain (*IPLA947*) isolated from an artisanal cheese (Cuesta