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Whey Starter for Grana Padano Cheese: Effect of Technological Parameters on Viability and Composition of the Microbial Community

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ABSTRACT

This work aimed to investigate the effects of thermal treatments and yeast extract addition on the composition of the microbial community of natural whey starters for Grana Padano cheese. Different natural whey starter samples were held at 4°C for 24 h (cooling treatment), or at –20°C for 24 h (freezing treatment) to evaluate the possibility of conservation, or at 54°C for 1 h (heat treatment) to evaluate the effect of the temperature commonly used during curd cooking. Separately, another set of samples was enriched with 0.3, 0.5, and 1.0% (wt/vol) of yeast extract to study its effect on the growth of lactic acid bacteria (LAB) in the starter. The new approach in this study is the use of 2 culture-independent methods: length heterogeneity (LH)-reverse transcription (RT)-PCR and fluorescence microscopy. These techniques allowed us to easily, quickly, and reproducibly assess metabolically active LAB in the control and treated samples. The LH-RT-PCR technique distinguished microorganisms based on natural variations in the length of 16S rRNA amplified by RT-PCR, as analyzed by using an automatic gene sequencer. Fluorescence microscopy counts were performed by using a Live/Dead BacLight bacterial viability kit. The repeatability of LH-RT-PCR showed that this technique has great potential to reveal changes in the microbial community of natural whey starters for Grana Padano cheese. All species showed low sensitivity to cold (4°C). However, after the freezing (–20°C) and heating (54°C) treatments, different behaviors of the species were reported, with significant changes in their viability and relative composition. Heating treatment during curd cooking profoundly affected the viability and composition of the community that remained in the cheese and that consequently modified the microbial population. At the same time, this treatment produced the selection of LAB in whey and could be considered as the first step in natural whey starter production. Addition of yeast extract stimulated the growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *lactis* to the detriment of *Lactobacillus helveticus* species. Because the yeast extract altered the microflora balance, whey starter conservation at –20°C and yeast extract addition cannot be suggested as technological innovations.
Key words: natural whey starter, Grana Padano cheese, length heterogeneity-reverse transcription-polymerase chain reaction, fluorescence microscopy

INTRODUCTION

The natural whey starter used in the production of Grana Padano cheese is a complex microbial association of lactic acid bacteria (**LAB**), not only because of the presence of various species, but also because of the large number of biotypes (Giraffa et al., 1997). It is obtained from the previous day's cheese-making whey (named sweet whey) incubated at a decreasing temperature, favoring the growth of a large number of viable thermophilic LAB (Mucchetti and Neviani, 2006). Different studies have been carried out to characterize the natural whey microflora, which are mainly composed of homofermentative thermophilic lactobacilli (e.g., *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis*) and a minor variable presence of heterofermentative lactobacilli (*Lactobacillus fermentum*) and *Streptococcus thermophilus* (Beresford et al., 2001; Mucchetti and Neviani, 2006). Understanding the dynamics of such communities is very laborious and time-consuming. Moreover, the inability to cultivate more than a small proportion of the bacteria that can be visualized by direct count procedures makes it difficult to obtain information on the entire microbial community (Head et al., 1998; Marsh 1999). Thus, to study interactions among microorganisms, it is important to study the ecosystem without dissociating it. Culture-independent approaches, such as those involving PCR, provide new insight by helping to define the biodiversity of communities and permitting the detection of viable, nonviable, and viable but noncultivable populations.

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Various PCR-based molecular typing methods have been developed for the analysis of populations, including denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, single-strand conformation polymorphism, and temporal temperature gel electrophoresis, that provide a rapid, yet good representative fingerprint of the microbial community structure without cultivation (Coppola et al., 2001; Ercolini et al., 2002; Duthoit et al., 2003; Hori et al., 2006). An increasing number of new methodologies incorporate automatic sequencing systems for laser detection of fluorescently labeled DNA fragments. The most common are terminal-restriction fragment length polymorphism (Marsh 1999; Osborn et al., 2000; Sánchez et al., 2006) and length heterogeneity-PCR (**LH-PCR**) analysis (Suzuki et al., 1998). Length heterogeneity-PCR analysis distinguishes different organisms based on natural variations in the length of 16S ribosomal DNA sequences (Ritchie et al., 2000). Fluorescent end-labeled PCR products are separated by capillary electrophoresis and detected by laser-induced fluorescence with an automated gene sequencer. The subsequent analysis of the relative amounts of amplified sequences originating from different microorganisms can then be made (Suzuki et al., 1998). Use of the LH-PCR method has been more limited than terminal-restriction fragment length polymorphism analysis in studies on microbial diversity. To date, it has been used to study microbial communities in aquatic environments (Suzuki et al., 1998), soil (Ritchie et al., 2000), and maize silage (Brusetti et al., 2006), and, in a few studies, on food-associated ecosystems such as dairy (Lazzi et al., 2004; Fornasari et al., 2006). Discriminating viable from dead cells is of importance in the development of bacterial detection methods. Recently, the effectiveness of the fluorescence microscopy technique to evaluate microbial viability was demonstrated in natural whey starter for Grana Padano cheese (Gatti et al., 2006). This technique allows the detection of microbial cells directly from their environment without any previous isolation step. Microbial cell viability is correlated with the physiological activity of a cell (Breeuwer and Abee, 2000). A disadvantage of DNA-based methods is that they do not distinguish between viable and nonviable cells. The DNA from lysed cells can persist for a long time in the environment; detection of RNA would help to determine the viability of the microorganisms because RNA is degraded rapidly upon cell death. Detection of 16S rRNA by reverse transcription-PCR (**RT-PCR**) is considered to be a useful indicator of viability (Bentsink et al., 2002; Wolffs et al., 2005). Ritchie et al. (2000) demonstrated that the LH-PCR technique was effective for measurement of the microbial community composition of soil.

This work aimed to investigate the effects of technological parameters on the microbial community of natural whey starter for Grana Padano cheese by using 2 culture-independent approaches directly on samples, avoiding classical undirected microbiological techniques. To test the reproducibility and suitability of the LH-RT-PCR method for assessing the microbial community structure in natural whey starters, we evaluated the variability at RT-PCR amplification and electrophoresis run levels.

A study of heating at cook temperature (+54°C) was done to investigate the effect on the microbial community during curd cooking as used in Grana Padano cheese manufacture. Study of cooling (+4°C) and freezing (–20°C) was performed to evaluate the possibility of conserving natural whey starter at low temperatures. Furthermore, the effect of adding different amounts of yeast extract to the sweet whey was evaluated with the aim of studying the possibility of using a growth stimulator.

MATERIALS AND METHODS

Natural Whey Starter Treatments

Ten natural whey starter samples from different Grana Padano dairies were analyzed. Immediately after collection, samples were cooled at 4°C, maintained in ice containers, and immediately after their arrival to the laboratory, were aliquoted and analyzed.

The following treatments were applied to several natural whey starters: 1) 3 samples were held at 4°C for 24 h (cooling treatment), 2) 2 samples were held at –20°C for 24 h (freezing treatment), and 3) 2 samples, previously brought to pH 6.4 with 10 *M* NaOH, were held at 54°C for 1 h (heat treatment) in a water bath. Moreover, 3 samples of sweet whey (i.e., a part of the whey collected from the vat immediately after curd extraction during the cheese-making process) were enriched with 3 different concentrations (0.3, 0.5, and 1%, wt/vol) of yeast extract (Oxoid, Garbagnate Milanese, Italy) as a microbial growth-stimulator factor. For natural whey starter preparation, sweet whey enriched samples, and not enriched samples used as a control, were incubated at 45°C until the acidity reached a value of 32°SH (Soxhlet-Henkel degrees)/50 mL. All whey cultures were subjected to viable cell counts by fluorescence microscopy and RNA extraction. Frozen whey starters (24 h at –20°C) were previously thawed at 37°C for 1 min.

Fluorescence Microscopy

Cell viability was estimated as indicated by Gatti et al. (2006). Fluorescence microscopy counts were performed by using a Leica DMSL fluorescent microscope (Leica Microsystems, Wetzlar, Germany) provided with a mercury vapor source (100 W) and a fluorescein isothiocyanate filter (Leica Microsystems). A Live/Dead *Bac-*

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