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Identification of a microscopically selected microorganism in milk samples[1]

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ABSTRACT

Identification of unwanted microbial contaminants microscopically observed in food products is challenging due to their low abundance in a complex matrix, quite often containing other microorganisms. Therefore, a selective identification method was developed using laser capture microdissection in combination with direct-captured cell PCR. This procedure was validated with *Geobacillus stearothermophilus* and further used to identify microbial contaminants present in some industrial milk samples. The microscopically observed contaminants were identified as mainly *Methylobacterium* species.
Key words: laser capture microdissection, cultivation-independent identification, bacterial contamination, direct-captured cell PCR

INTRODUCTION

The presence of bacterial contamination in food products represents a serious health threat to consumers worldwide (Tauxe et al., 2010). Microbial growth has a negative effect on product integrity and quality (i.e., the product fails to meet the customer expectations by the presence of unacceptably high numbers of microorganisms, spoilage, or contamination with pathogens and or microbial toxins). Microbial contamination can originate from the raw materials but also from microorganisms housed in a biofilm on the processing equipment (Brooks and Flint, 2008; Van Houdt and Michiels, 2010). It is, therefore, of primary importance to identify and eliminate contaminants from a health and economical point of view.

Microscopy is still the gold standard for observing (and counting) bacteria in raw milk and other dairy products as a microbial quality control. It has proven to be a valuable, rapid, and reliable quality control routine tool used in the dairy industry. However, traditional identification methods for microbial contaminants rely on phenotypic identification using cultivation-based methods (European Directorate for the Quality of Medicines and HealthCare, 2013) and biochemical assays (Smith et al., 1972). These methods suffer from 3 major drawbacks. First, they can only be used for organisms that can be cultivated in vitro. This approach requires pure culture of the microorganism (i.e., a laboratory culture containing a single species derived from a mixed culture with many species), which takes, in the best case, 48 h due to the time necessary for dilutions, platings, and incubations. Second, heterogeneous samples make it difficult to give unambiguous results. And third, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. The most promising strategy for the classification leading to reliable identification would include as a first step the analysis of the 16S rRNA gene (Olsen et al., 1986). However, preferential lysis of cells before a PCR-based approach will most likely bias the view of the composition of the microbial diversity in the sample (von Wintzingerode et al., 1997). Therefore, it is important to develop a method that can selectively isolate microorganisms for further identification in a cultivation-independent manner.

Single-cell gene/genome sequencing workflows are based on the physical isolation of individual cells and the use of these cells as templates for PCR. Single cells can be isolated via (1) micromanipulations (e.g., micropipette; Ishøy et al., 2006; Kvist et al., 2007), suffering

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from limited throughput as well as difficulties when working in complex growth or storage media (e.g., food matrices); (2) flow cytometry fluorescence-activated cell sorting (Müller and Nebe-von-Caron, 2010), where prior knowledge is necessary for staining (selective fluorophores); and (3) laser capture microdissection (**LCM**) for the microscopic selection of the microorganism of interest. Laser capture microdissection allows the selective isolation of cells under direct microscopic visualization by the transfer of laser energy to a thermolabile layer, which forms a polymer-cell composite (Emmert-Buck et al., 1996), followed by the removal of the cell from the heterogeneous sample. A few publications report the use of LCM for the identification of microorganisms in histological tissues, using fluorescently labeled oligonucleotide probes or primers (Ryan et al., 2004; Klitgaard et al., 2005; Wang et al., 2010; De Hertogh et al., 2012), as well as in complex environmental samples (Gloess et al., 2008; Yanagihara et al., 2011).

We developed an LCM-based approach for the identification of specific bacteria in dairy samples based on atypical morphology, starting from a limited number of cells. The bottlenecks that had to be overcome were the smaller cell sizes of microorganisms, lower amount of DNA, and the number of isolated cells necessary for further analysis with DNA, RNA, or proteins, which traditionally ranges between 100 and thousands of cells (Espina et al., 2006). Milk samples from a food industrial plant where an unidentified bacterium was present were then obtained and analyzed.

MATERIALS AND METHODS

Specimens

Geobacillus stearothermophilus was purchased from the Belgian Coordinated Collections of Microorganisms/Laboratory for Microbiology of the Faculty of Sciences of Ghent University (BCCM/LMG) bacterial collection (LMG11163; Ghent University, Ghent, Belgium). The strain was grown at 55°C overnight in brain-heart infusion (BHI) medium and then harvested for further processing. The culture grown *G. stearothermophilus* was mixed with a milk sample (1:4 culture:milk) and embedded in agarose to make microscopic slides. The industrial milk sample was embedded in agarose as well, without prior manipulations.

Sample Processing, Microtome, and Staining

First, 500 μL of sample was added to 500 μL of 1% agarose and vortexed immediately for at least 10 s. The tube was placed at 4°C before the preparation of microscopic slices. The solidified agarose-embedded sample was transferred into an embedding holder. The holder was placed into an STP420 Tissue Processor (Thermo Scientific, Erembodegem, Belgium), which gradually dehydrated the sample (using an increasing alcohol series followed by isopropanol and xylene; all from Sigma-Aldrich, Diegem, Belgium) and embedded it in paraffin. Up to ten 8-μm-thick sections were prepared from each sample using presterilized microtome blades. The sections were then placed on gelatin-coated microscopic glass slides (25 × 75 mm) and incubated on a hot plate (60°C) for 1 h to allow the paraffin to melt onto the slide. For the staining procedure, the slides were dewaxed and hydrated in succeeding baths of xylene (3 × 5 min), isopropanol (1 × 5 min), and a decreasing alcohol series (10 s in 95, 80, 70, 50, and 0% alcohol, successively). Then, they were stained in 0.1% (mass/vol) aqueous methylene blue dye and dehydrated with an increasing alcohol series (10 s in 0, 50, 70, 80, and 95% alcohol), isopropanol (2 × 5 min), and xylene (3 × 5 min) and finally air-dried, while microbial contamination was prevented.

LCM

Laser capture microdissection was performed using the Arcturus PixCell II laser capture microscope (Life Technologies, Gent, Belgium). All slides were viewed at a 400× magnification. Hand gloves were used throughout the whole LCM procedure to prevent contamination of the slides. The CapSure HS-LCM caps (Life Technologies) were pretreated with 30-min UV exposure at a maximum distance of 10 cm to neutralize potentially present DNA as polymerase template (Champlot et al., 2010). The cells of interest were isolated on the caps by applying a laser pulse. The magnitude of the laser pulse (i.e., spot size, intensity, and duration) was optimized to accurately remove the cells from the slide. For isolation of individual bacteria, the spot size was kept minimal (i.e., 7.5 μm), with an intensity of 60 mW (±30) and a duration of 4 ms (±1). After the desired cells were captured, a sterile 0.5-mL tube was placed over the cap surface. The assembly was stored at room temperature.

PCR: Amplification of the 16S rRNA Gene

The thermolabile polymer on top of the cap, including the captured bacteria, was scratched off in a laminar flow hood with a sterile pipette tip and immediately submerged in the PCR mix. The primers used for amplification of the 16S RNA gene are listed in Table 1 (b16SF and 1495R). All primers were purchased from Life Technologies.

Prior to amplification, the PCR solutions and consumables were first decontaminated according to the