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The microbial content of raw and pasteurized cow milk as determined by molecular approaches

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ABSTRACT

The microbial composition of raw and pasteurized milk is assessed on a daily basis. However, many such tests are culture-dependent, and, thus, bacteria that are present at subdominant levels, or that cannot be easily grown in the laboratory, may be overlooked. To address this potential bias, we have used several culture-independent techniques, including flow cytometry, real-time quantitative PCR, and high-throughput DNA sequencing, to assess the microbial population of milk from a selection of commercial milk producers, pre- and postpasteurization. The combination of techniques employed reveals the presence of a previously unrecognized and diverse bacterial population in unpasteurized cow milk. Most notably, the use of high-throughput DNA sequencing resulted in several bacterial genera being identified in milk samples for the first time. These included *Bacteroides*, *Faecalibacterium*, *Prevotella*, and *Catenibacterium*. Our culture-independent analyses also indicate that the bacterial population of pasteurized milk is more diverse than previously appreciated, and that nonthermoduric bacteria within these populations are likely to be in a damaged, nonculturable form. It is thus apparent that the application of state-of-the-art approaches can provide a detailed insight into the bacterial composition of milk and could potentially be employed in the future to investigate the factors that influence the composition of these populations.
Key words: high-throughput DNA sequencing, flow cytometry, quantitative PCR

INTRODUCTION

Milk harbors a complex microbial community, including microorganisms of industrial importance, that possess health-promoting features and that are of concern from a food quality or safety perspective. Thus, the milk microbiota is the focus of constant attention. Such testing occurs daily on both raw and pasteurized milk and is governed by a variety of methods and standards across different jurisdictions. The microbial composition of milk is influenced by several different parameters such as, in the case of raw milk, the microorganisms present in the teat canal, on the surface of teat skin, in the surrounding air, in feed, as well as other environmental factors including housing conditions, the quality of the water supply, and equipment hygiene (Verdier-Metz et al., 2009, 2012; Vacheyrou et al., 2011; Braem et al., 2012). The microbiota of pasteurized milk is thought to be determined by the percentage of thermoduric bacteria that survive pasteurization temperatures and by the bacteria associated with postpasteurization contamination, which include psychrotrophic bacteria, such as *Pseudomonas* (Ternström et al., 1993; Fromm and Boor, 2004). The techniques used to identify and count the bacterial populations present generally involve culturing on agar media and are labor intensive and time consuming. Furthermore, microorganisms that cannot be easily cultured in the laboratory, or are present as subdominant populations, are not detected using these approaches (Paszyn'ska-Wesołowska and Bartoszcze, 2009). Indeed, comparative culture-based and culture-independent (flow cytometry) analysis of identical milk samples have provided significantly different results (Gunasekera et al., 2002). Other culture-independent techniques, and in particular those which are DNA-based, provide a means of examining the bacterial composition of milk without introducing culture-based biases. These DNA-based approaches have included denaturing gradient gel electrophoresis and single stranded conformation polymorphisms. These allow a comparison of the relative diversity of different bacterial populations and can, to some extent, reveal the identity of specific components (Callon et al., 2007; He et al., 2009). Methods, such as quantitative real-time PCR (**qPCR**), have increasingly been employed, which permit rapid identification and quantification, albeit only of specific target microbes

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(Rodríguez-Lázaro et al., 2005; He et al., 2009). Even more recently, significant developments have occurred in the field of microbial ecology as a consequence of the development of culture-independent analysis via high-throughput DNA sequencing. This approach can provide a more in-depth insight into the diversity and dynamics of entire microbial communities (Sogin et al., 2006; Andersson et al., 2008; Quigley et al., 2011; Delmont et al., 2012). In a few exceptional cases this technology has been applied to dairy-based environments, such as cheese (Masoud et al., 2011; Alegría et al., 2012; Quigley et al., 2012b) and kefir (Dobson et al., 2011). The aim of this study was to provide a detailed insight into the microbial composition of raw and pasteurized milk, sourced from a variety of facilities using high-throughput DNA sequencing in combination with other culture-independent approaches.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains used in this study were *Lactococcus lactis* HP and *Pseudomonas aeruginosa* PAO-1 (Teagasc Food Research Centre Culture Collection). *Lactococcus lactis* was grown in M17 broth with glucose (GM17) at 30°C and *P. aeruginosa* was grown in Luria broth (**LB**) and on 1% LB agar plates at 37°C.

Flow Cytometry

The viability of microbial populations found in raw and pasteurized milk samples obtained from the Teagasc Research Centre, Moorepark, dairy herd were investigated using flow cytometry. Flow cytometry analyses were performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using 2 air-cooled lasers, a 20-mW solid state laser (emission, 488 nm) and a 17 mW HeNe laser (emission, 633 nm), and 5 sensors for the detection of forward and sideward light scatter: green (525 nm), yellow (575 nm), and far red (695 nm) fluorescence. Flow cytometry detector and threshold settings were established using a series of control studies. Here, raw milk (from the Moorepark dairy herd) spiked with *L. lactis* were subjected to heat treatments at 80°C for 30 min and 3 h. Prior to analysis, proteins and lipids were removed from milk samples using a modified version of the procedure described by (Gunasekera et al., 2000). Briefly, milk was treated with 20 arbitrary units/mL of proteinase K (Sigma-Aldrich Co., Arklow, Ireland), and 500 μL of 0.05% Triton X-100 (Sigma-Aldrich Co.) at 37°C with shaking for 45 min. Samples were centrifuged at 17,000 × *g* at room temperature for 10 min, following which the milk fat layer and supernatant were removed. The resulting pellet was washed twice and resuspended in 1 mL of filtered (0.22 μm) PBS (Invitrogen, Life Technologies, Carlsbad, CA). Viability testing was performed using BD Cell Viability Kit (BD Biosciences). Samples were stained with propidium iodide (41 n*M*) for 10 min on ice, followed by thiazole orange staining (8.5 μ*M*) for 15 min in the dark. Cell samples were delivered at the low flow rate, corresponding to 500 to 1,000 cells/s, until 10,000 cells were measured. Fluorescence signals were recorded by using the detector settings forward scatter (FSC) = 300; side scatter (SSC) = 300; fluorescein isothiocyanate (FITC) = 600; and propidium iodide = 500. A threshold was set at an SSC signal of 300 to reduce background noise deriving from cellular debris and traces of milk components remaining following treatment. Following the generation of threshold parameters, commercial raw and pasteurized milk samples were assayed to assess the relative proportion of live-to-dead microbes. Data analysis was performed using the FACSDiva software v.5.0.2 (BD Biosciences).

Collection and Treatment of Milk Samples

Cow milk, both pre- (i.e., raw) and postpasteurization, were obtained from 6 industrial facilities around Ireland; 3 samples of each milk type were collected for analysis. Fresh unpasteurized milk was also obtained from the dairy herd at Teagasc Research Centre, Moorepark, and was pasteurized in-house using a Microthermics Heat exchanger (Microthermics, Wellington, CT) at 72°C for 15 s followed by rapid cooling to 4°C. All milk samples were transported to the laboratory on ice before storage at −20°C. Milk samples were defrosted at 4°C before use.

High-Throughput Sequencing and Bioinformatics Analysis

Prior to extraction of DNA, milks (both raw and pasteurized) were treated with 100 μg/mL of nucleic acid stain ethidium monoazide (EMA; VWR, Dublin, Ireland; Rudi et al., 2005) to inactivate DNA not associated with living microbes (see supplementary material). Total DNA was then isolated from 1 mL of each raw and pasteurized milk sample using the PowerFood Microbial DNA extraction kit (MO BIO Laboratories, Carlsbad, CA) in accordance with manufacturer's instructions. Additionally, a 10-min incubation step at 70°C was incorporated to improve DNA yield, as described previously (Quigley et al., 2012a). The DNA extracts were used as a template for PCR amplification of 16S rRNA tags (V4 region; 239 nucleotides long) using universal 16S rRNA-targeting primers predicted