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High-throughput metataxonomic characterization of the raw milk microbiota identifies changes reflecting lactation stage and storage conditions

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ABSTRACT

Low temperature is used to control the growth of bacteria in milk, both pre- and post-pasteurization. As the duration of refrigerated storage extends, psychrotrophs dominate the milk microbiota, that can produce heat stable lipases which negatively impact the organoleptic qualities of milk. Here we examine the influence that refrigeration temperature (2 °C, 4 °C and 6 °C) and storage duration (96 h) have on the microbiota composition (16S profiling) of raw bulk tank milk (BTM). To reflect a proposed change to current farming practices, raw milk was blended after each milking (8 milkings) and stored for five consecutive days in each temperature-specific tank. Here 16S rRNA-based microbiota compositional analysis was performed after milk was collected on day 1 and again after the final addition of milk at day 5. In addition to assessing the impact of the duration and temperature of storage, the influence of lactation stage, i.e. mid- versus late-lactation, on the microbiota of the blended BTM was also examined. Overall, both temperature and length of storage had surprisingly little influence on the raw milk microbiota, other than an increase in proportions of *Gammaproteobacteria* in the blended milk samples collected after pooling on day 5, and in samples stored at 6 °C. However, lactation stage had a considerable influence on microbiota composition, with milk from mid-lactation containing higher proportions of *Bacteroides*, *Faecalibacterium*, *Campylobacter* and *Rhodanobacter*, and late-lactation milk containing higher proportions of Actinobacteria. Overall, the study demonstrates that current temperature and storage duration practises impact the microbiota of raw milk, but these impacts are modest relative to the more considerable differences between mid and late-lactation milk.

1. Introduction

The microbiota of raw milk is complex (Quigley et al., 2013b), and its composition, which is influenced by a multitude of intrinsic and extrinsic factors, is an important consideration for milk producers, processors and consumers. Indeed, the microbiota of milk influences the subsequent production of a wide variety of dairy products, such as cheese, butter, yogurt and dairy powders, and can contribute to the quality and safety of these foods (McInnis et al., 2015). Dairy producers therefore need to be aware of the influence of environmental factors, such as lactation period (McInnis et al., 2015; O'Connell et al., 2016) and storage conditions, such as temperature and duration of storage, on the microbial composition of raw milk (O'Connell et al., 2016).

Currently, most of what is understood about the presence of undesirable microorganisms in milk has been elucidated from selective plate cultivation-based techniques. These culture-based assays reveal the presence or absence of specific groups of bacteria, based on their phenotype (Quigley et al., 2013b). These phenotypic assays, which are most commonly utilised by the dairy industry, target bacteria that proliferate during cold storage (psychrotrophs) or survive heat treatments (thermoduric bacteria including spore-formers). Psychrotrophic populations, which may increase during storage at refrigeration temperatures, include *Pseudomonas* and *Acinetobacter* spp. (Quigley et al., 2013b; Raats et al., 2011). These populations are of particular significance as they are primarily responsible for spoilage of refrigerated dairy products (Machado et al., 2013; Raats et al., 2011), most frequently through the production of heat stable lipases which can survive heat treatments designed to eliminate psychrotrophic bacteria

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(Andersson et al., 1979; Sørhaug and Stepaniak, 1997). Thermoduric bacteria are also of concern due to their spoilage and toxigenic potential (Doyle et al., 2015).

Recently, it was found by culture-based surveillance that the microbial quality of blended raw milk stored at refrigeration temperatures (2, 4 or 6 °C) was not significantly altered by storage time (O'Connell et al., 2016). However, a corresponding study that focused on lactation stage revealed that it has a more considerable influence, with total bacterial counts (TBCs) being higher in late lactation milk (O'Connell et al., 2015), which, in the Irish dairy farm system, corresponds to winter. These studies are of significant applied value because longer raw milk collection interval extensions are more practical for milk processors, storage at higher temperatures is more economic for milk producers, and reductions in the quality in late lactation milk can influence its downstream use. Despite the potential value of these findings, it is important to note that culture-based methods are ultimately limited to revealing what can be grown in laboratory conditions, which may represent only a fraction of the bacteria present in the environment (Hugenholtz and Pace, 1996; Ward et al., 1992). Advances in DNA-based technologies and, more specifically, the application of next generation sequencing has provided a greater insight into the microbiota composition of milk and dairy products (Ercolini et al., 2009; Quigley et al., 2013a; Raats et al., 2011; Vacheyrou et al., 2011; Verdier-Metz et al., 2009). This type of molecular analysis was initially developed for environmental microbiology but is equally applicable to the analysis of raw milk and other dairy products (Gschwendtner et al., 2016; Mallet et al., 2012; McInnis et al., 2015; Quigley et al., 2013a; Quigley et al., 2016; Thierry et al., 2005; Walsh et al., 2016; Wolfe et al., 2014)This present study was run concurrently with O'Connell and colleagues (O'Connell et al., 2016). As such, the conditions and experimental design described here are identical as that study, with the exception of the way in which samples were processed for analysis and the goal of the study. The goal of this study was to characterise the raw milk microbiota using high-throughput sequencing, while O'Connell and colleagues targeted a subset of cultivable microbes. Here, we address the important issues of storage duration, storage temperature and lactation period on the microbial content of raw milk using high-throughput metataxonomic analysis.

2. Methods

2.1. Experimental design

The study was conducted at the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Cork, Ireland, using milk produced from spring-calved dairy cows, as described previously by O'Connell and colleagues (Ercolini, 2013). Milk production over two 6-week periods was studied; period 1 extended from August 11 to September 26, corresponding to mid-lactation, and from October 13 to November 21, corresponding to late-lactation. During period 1 and the first 4 weeks of period 2, the cows were outdoors consuming a diet of grass. During the remaining 2 weeks of period 2, the cows were housed indoors during times of heavy rainfall on cubicles fitted with rubber mats that were bedded with lime, and they consumed a diet consisting of approximately 50% grazed grass and 50% grass silage. Teats were disinfected prior to milking as described previously (O'Connell et al., 2016). Two milking's were conducted daily for the duration this study. Upon the completion of each milking, equipment was sanitized as described previously (O'Connell et al., 2016). Three identical 4000 L bulk tank units (Swiftcool, Dairymaster) were used in this study. The 3 bulk tanks were set to cool milk to the different temperatures at the beginning of each test period. Valves in the milkline were used to divide the milk flow in equal proportions (300 L into each tank at each milking) to each of the 3 tanks. The milk passed through a plate cooler and was cooled to approximately 14.5 °C before entering each tank. The milk was subsequently cooled to the desired temperature, 2, 4 or 6 °C, within the tank. Upon completion of the 96-h storage period, each bulk tank was sterilised as described previously (O'Connell et al., 2016).

Equal volumes of milk were pumped (300 L) into each tank at each milking for four days (n = 8 milkings) each week, for two 6-week periods, representing mid and late-lactation milk, respectively, and each tank was set at a different temperature (2, 4 or 6 °C) at the beginning of each week. Each treatment was applied to each tank on two occasions within each period. Milk was collected aseptically from each bulk tank after the morning milking on day 1 and on day 5 (representing 96 h of storage) using sterile blue dippa collection bottles (OCON Chemicals, Ireland). The latter represented a mixture of all milk collected over the five day period and was investigated to assess the consequences of extending milk collection intervals at farms.

2.2. DNA extraction

For each sample 15 mL of raw milk was centrifuged at 5444 × *g* for 30 min at 4 °C. The fat layer was carefully removed and the supernatant was decanted. Cell pellets were then homogenised in 90 μL lysozyme solution 50 mg/mL (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) and 50 μL of 50 U/mL mutanolysin (Sigma Aldrich, Arklow, Co. Wicklow, Ireland), vortexed and incubated at 55 °C for 15 min vortexed at 2–3 min intervals. Then 28 μL of proteinase K solution (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) was then added to the cell pellet homogenate and the samples were incubated at 55 °C for 15 min. After incubation samples were centrifuged at 14, 000 × *g* for 5 min, supernatant was removed and the PowerFood DNA isolation kit was used as per manual (Mobio, Carlsbad CA) (O'Sullivan et al., 2015). DNA was quantified and quality checked by gel electrophoresis and Nanodrop 1000 instrument (Thermo Fisher Scientific, Inc.).

2.3. Quantitative PCR

Quantitative PCR (qPCR) was carried out on samples to quantify the total bacteria in each sample. This qPCR was carried out as per (Fouhy et al., 2012), except for the use of Kapa SYBR fast. Standards, samples and negative controls were all run in triplicate.

2.4. 16S rRNA amplicon preparation and high throughput sequencing

The V3–V4 variable region of the 16S rRNA gene was amplified from DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). PCR reactions were completed on the template DNA. Initially, the DNA was amplified with primers specific to the V3–V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Walsh et al., 2016). Samples were sequenced on the MiSeq sequencing platform in the Teagasc sequencing facility, using a 2 × 300 cycle V3 kit, following standard Illumina sequencing protocols.

2.5. Bioinformatic and statistical analysis

Three hundred base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (Caporaso et al., 2010). Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release 111. Samples were then rarefied to an even depth of sequences per sample. Alpha and beta diversities were