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Microbial diversity of consumption milk during processing and storage

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ABSTRACT

Bovine milk contains a complex microbial community that affects the quality and safety of the product. Detailed knowledge of this microbiota is, therefore, of importance for the dairy industry. In this study, the bacterial composition of consumption milk was assessed during different stages in the production line and throughout the storage in cartons by using culturing techniques and 16S rRNA marker gene sequencing. Monthly samples from two dairies were analyzed to capture the seasonal variations in the milk microbiota. Although there was a core microbiota present in milk samples from both dairies, the composition of the bacterial communities were significantly influenced by sampling month, processing stage and storage temperature. Overall, a higher abundance of operational taxonomic units (OTUs) within the order *Bacillales* was detected in samples of raw and pasteurized milk from the spring and summer months, while *Pseudomonadales* and *Lactobacillales* OTUs were predominant in the winter months. OTUs belonging to the order *Lactobacillales, Pseudomonadales, Clostridiales* and *Bacillales* were significantly more abundant in milk samples taken immediately after pasteurization compared to raw milk samples. During storage of milk in cartons at 4 °C, the bacterial composition remained stable throughout the product shelf life, while storage at 8 °C significantly increased the abundance of OTUs belonging to the genus *Bacillus* and the plate count levels of presumptive *Bacillus cereus*. The knowledge obtained in this work will be useful to the dairy industry during their quality assurance work and risk assessment practices.

1. Introduction

Bovine milk is a nutritious food product consumed as a beverage or used as an ingredient in the production of a wide range of dairy and non-dairy products. Raw milk contains highly diverse bacterial populations, some of which such as lactic acid bacteria, are beneficial for milk processing, and others, such as spore-forming and psychrotrophic bacteria, are involved in spoilage and disease (as reviewed by Quigley et al., 2013a). Microbial contamination in the dairy chain may occur on the farm, during transport and in the processing facilities. Therefore, the composition of the raw and pasteurized milk microbiota is greatly influenced by several factors, such as farm management practices, seasons and hygienic practices and storage conditions throughout the value chain (Mallet et al., 2012; Quigley et al., 2013a; Vithanage et al., 2016).

To avoid milk spoilage and ensure safe products, the dairy industry applies thermal treatment to reduce the microbial load. However, spore-formers and other heat-resistant bacteria may survive this treatment (Christiansen et al., 2006; Novak et al., 2005). The storage of processed milk products at an optimal temperature is also important to minimize microbial growth and to reduce enzymatic activities, thereby maintaining the intended product shelf life. Previous studies have shown that the cold chain temperature is not always kept within the recommended range, particularly in consumer refrigerators, which increases the risk of spoilage and growth of potential pathogenic microorganisms in food products during storage (Rossvoll et al., 2014; Schmidt et al., 2012).

To ensure delivery of high quality products with long shelf life, the dairy industry needs to evaluate milk quality throughout the value chain. Traditionally, culture-dependent techniques have been used to assess the milk microbiota, both in research and industry (Raats et al., 2011). However, these methods are usually time consuming and labor intensive and may not provide a realistic picture of the diversity of the microbial communities. Recent advances in genomic technologies and bioinformatics tools have enabled characterization of the total microbiota directly from food matrices (Ercolini, 2013). By using culture-independent methods, such as high throughput sequencing (HTS), bacterial genera not previously associated with milk, e.g., *Bacteroides*,

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Parabacteroides and *Faecalibacterium*, have now been detected in cow milk (Quigley et al., 2013b). Also, while *Pseudomonas* spp. appeared to be eliminated by pasteurization as detected by plate counts, culture-independent analyses of the same samples showed a reduction in bacterial levels rather than an elimination (Quigley et al., 2013b).

Knowledge of the actual composition of the bacterial community in milk throughout the value chain will facilitate the dairy industry in their production of safe and high-quality products in a long-term sustainable manner. In Norway, a large-scale study on contamination routes in the dairy industry was performed in 1995–1998 using plate count techniques (Eneroth et al., 1998; Svensson et al., 1999). However, no systematic survey on the composition of the milk microbiota throughout the Norwegian dairy value chain has been conducted in recent years. In the current work, the microbial composition of milk samples from two dairies was characterized. Samples were taken from raw milk in silo tanks through processing at the dairy plant, and during storage at 4 °C and 8 °C until end of product shelf life. In addition, total aerobic bacterial counts and presumptive *Bacillus cereus* levels (hereafter designated *B. cereus*), which are commonly used microbiological parameters in industry today, were determined by traditional culturing techniques.

2. Materials and methods

2.1. Milk sampling and DNA extraction

The bovine milk samples (n = 864) included in this experiment were collected during a 13-month period (from June 2015 to June 2016) from two dairies (A and B) situated in two different geographical locations in Norway. Dairy A is a large-scale processing facility (annual production > 80 million L of milk), while Dairy B produces substantially less milk (annual production < 40 million L of milk). Each month, with the exception of December 2015, the following samples were collected successively from each dairy: raw bovine milk from silo tanks (100 mL), homogenized and pasteurized (72 °C for at least 15 s) full-fat milk from intermediate storage tanks (100 mL), and 15 one-liter cartons produced the same day. Milk from the same silo and intermediate storage tanks, where the temperature was kept at 3–4 °C, were sampled throughout the trial period. During transport to the laboratory, the milk samples were kept cold (maximum 4 °C) and the transport time did not exceed 1.5 h. The samples were held at 4 °C at the laboratory until the next day, when culturing and DNA extraction took place. Samples from the silo tanks, the intermediate storage tanks and three of the pasteurized milk cartons were analyzed at Day 1 after sampling, while the remaining cartons were kept at 4 °C or 8 °C for 6–8 days (mid-shelf life) or 13–14 days (end of shelf life reported on the milk cartons) before processing. Three replicates of each sample from each silo tank and intermediate storage tank were analyzed, while three cartons, with two replicate samples from each, were analyzed for every temperature and time point. DNA was extracted from 10 mL of milk from each sample as previously described by Porcellato et al. (2016) and stored at − 20 °C until further analysis. For all samples analyzed, milk samples containing 15% glycerol were stored at − 80 °C as back-up material.

2.2. Culture conditions

The total aerobic plate counts were determined for all milk samples on Plate Count Skimmed Milk Agar (Merck KGaA, Darmstadt, Germany), according to ISO 4833-1:2013 (Anon., 2013). Enumeration of viable *B. cereus* isolates from milk cartons was performed on *B. cereus* selective agar with supplements according to NMKL method No. 67 (Nordic Committee on Food Analysis), but without additional plating on blood agar plates. All culture media were obtained from Oxoid Ltd., Hampshire, England. Frozen back-up samples were used for bacterial enumeration in two samplings from Dairy B, July 2015 (total aerobic colony counts at end of shelf life at 4 °C) and February 2016 (*B. cereus* colony counts at end of shelf life at 8 °C). The impact of freezing on the total aerobic and *B. cereus* colony counts was tested by plating previously frozen and fresh replicas of the same milk sample. This test revealed that freezing had no or very little effect on the colony counts. Bacterial levels in raw milk (from the total aerobic plate counts) were analyzed by two-way ANOVA model using the sampling month and dairy (A and B) as factors. Bacterial levels in pasteurized milk (total aerobic plate count and *B. cereus* colony count) were analyzed using a linear cross design model including dairy, sampling month and storage temperature as fixed factors and sampling day as a covariate. The model also included the interaction between temperature and the covariate. All statistical tests were performed with R statistic version 3.3.0 (R Core Team, 2017).

2.3. 16S marker gene sequencing and bioinformatics analysis

The microbial composition of all milk samples (n = 864) was studied according to the method described by Porcellato et al. (2016), with minor changes. In brief, the variable region V3 and V4 of the bacterial 16S rRNA gene was amplified using the universal primers Uni340F (5′-CCTACGGGRBGCASCAG-3′) and Bac806R (5′-GGACTACYVGGGTATCTAAT-3′) (Takai and Horikoshi, 2000). The PCR reaction was performed using 4 μL of DNA. 20 μL of the resulting PCR product was purified using 0.8 × of Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions. Five μL of the purified PCR product was used as template for the second PCR using customized primers with unique sample barcodes (Table S1). Library normalization was performed using the Sequal-Prep™ Normalization plate (Thermo Fischer Scientific, Oslo, Norway) and quantified using the PerfeCTa NGS quantification kit (Quanta Biosciences, Beverly, MA, USA). Sequencing of the library was done using the Illumina Miseq platform (Illumina, San Diego, CA, USA) with a 300 bp paired-end sequencing kit. Raw Illumina fastq files were merged and quality filtered using the Usearch 8.0 (Edgar, 2010). Only sequences with lengths over 380 base pairs were kept for further analysis. The resulting files were demultiplexed using Qiime 1.9.0 (Caporaso et al., 2010). The Usearch operational taxonomic unit (OTU) picking algorithm (Edgar, 2013) with clustering at 97% pairwise identity was chosen as OTU picking method. Singletons and chimeras sequences were removed before the clustering step as part of the Usearch pipeline. The taxonomy was assigned to each OTU as previously described (Porcellato and Skeie, 2016). Sequences were deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB23297.

2.4. Statistical analysis of the microbial community

The final OTU table was normalized using the cumulative-sum scaling method to account for different sequencing depth (Paulson et al., 2013). This method has previously been shown to perform similarly or better compared to other methods of normalization (Kable et al., 2016; Paulson et al., 2013). Alpha diversity (within-sample diversity) was calculated with the R-package Vegan (Oksanen et al., 2017). Richness and diversity indexes were calculated with the Chao1 and Shannon index, respectively. Beta diversity (between-sample diversity) was estimated using both the weighted and unweighted Unifrac distance between the milk samples. Nonmetric dimensional scaling (NMDS) was computed from the weighted Unifrac distance matrix using the R-package Vegan. The weighted Unifrac distance matrix was also used to compute the permutational multivariate analysis of variance (Adonis function in R-package Vegan) using the different experimental factors (dairy plant, month, temperature, day and sample type). Differential abundances for each OTU between sample groups were determined using the zero-inflated log-normal mixture model (available in the metagenomeSeq R package, http://bioconductor.org/packages/release/bioc/html/metagenomeSeq.html) after filtering the OTU table