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Characterization of the indigenous microflora in raw and pasteurized buffalo milk during storage at refrigeration temperature by high-throughput sequencing

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ABSTRACT

The effect of refrigeration on bacterial communities within raw and pasteurized buffalo milk was studied using high-throughput sequencing. High-quality samples of raw buffalo milk were obtained from 3 dairy farms in the Guangxi province in southern China. Five liters of each milk sample were pasteurized (72°C; 15 s); and both raw and pasteurized milks were stored at refrigeration temperature (1–4°C) for various times with their microbial communities characterized using the Illumina Miseq platform (Novogene, Beijing, China). Results showed that both raw and pasteurized milks contained a diverse microbial population and that the populations changed over time during storage. In raw buffalo milk, *Lactococcus* and *Streptococcus* dominated the population within the first 24 h; however, when stored for up to 72 h the dominant bacteria were members of the *Pseudomonas* and *Acinetobacter* genera, totaling more than 60% of the community. In pasteurized buffalo milk, the microbial population shifted from a *Lactococcus*-dominated community (7 d), to one containing more than 84% *Paenibacillus* by 21 d of storage. To increase the shelf-life of buffalo milk and its products, raw milk needs to be refrigerated immediately after milking and throughout transport, and should be monitored for the presence of *Paenibacillus*. Results from this study suggest pasteurization should be performed within 24 h of raw milk collection, when the number of psychrotrophic bacteria are low; however, as *Paenibacillus* spores are resistant to pasteurization, additional antimicrobial treatments may be required to extend shelf-life. The findings from this study are expected to aid in improving the quality and safety of raw and pasteurized buffalo milk.

Key words: high-throughput DNA sequencing, buffalo milk, refrigeration storage, indigenous microflora

INTRODUCTION

China ranks third in the world with regard to its number of dairy buffalo herds and the production of buffalo milk. Within the subtropical south of China, the buffalo farming system has traditionally consisted of both commercial-scale and individual farms from which raw milk is pooled and stored at refrigeration temperature (1–4°C) for up to 24 h before being transferred to dairy plants. This results in raw milk being stored for between 24 and 48 h before processing and pasteurization. Although low storage temperatures (≤4°C) reduce the growth of many bacteria, they can select for the growth of psychrotrophic bacteria known to cause spoilage of bovine milk (Champagne et al., 1994; DeJonghe et al., 2011; Reche et al., 2015). In addition, pasteurization is used to reduce the microbial load of milk, but the lysis of psychrotrophic bacteria may lead to the release of heat resistant proteolytic and lipolytic enzymes that could affect the shelf life of milk and dairy products such as cheeses and milk powders. A previous study showed that heat-resistant proteolytic enzymes from psychrotrophic bacteria could result in the degradation of cheese caseins, resulting in a reduced cheese yield (Mankai et al., 2012); whereas another report showed that raw goat milk should not be stored for more than 3 d at 4°C after heat treatment or the presence of lipolytic enzymes will reduce the quality of milk powder, which typically has a shelf-life of 180 d (Fonseca et al., 2013). In addition, gram-positive spore-forming bacteria are also known to contribute to milk spoilage, as they may survive heat treatments and become a dominant population within the microflora of pasteurized milk (Ranieri et al., 2009).

Microbial populations in raw milk are important for the quality of resulting dairy products. Culture-independent sequencing technology has been widely used

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in the analysis of microbial diversity within raw milk and dairy products, and could be used to determine the presence of harmful and beneficial bacteria (Delgado et al., 2013; Weber et al., 2014). The microflora of raw milk has been reported to change depending on the location of the herd, breeding practices, and lactation period. The specific composition of the milk microbiota directly affects the subsequent development of dairy products. However, the microbiota within raw and pasteurized Chinese water buffalo milk during refrigerated storage (≤4°C) has not been investigated. Therefore, the aim of the current study was to analyze shifts within the bacterial communities in raw and pasteurized buffalo milk during prolonged storage at refrigeration temperature and determine the dominant populations that may affect the quality and safety of the milk and resulting dairy products.

MATERIALS AND METHODS

Collection and Treatment of Milk Samples

Raw buffalo milk was obtained from 3 different dairy farms, with herds ranging from 50 to 1,000 buffalo. Farms were all located within the Guangxi province of China, ensuring that buffalo herds were raised in a similar subtropical climate with an average annual temperature of 21.6°C (70.9°F) and an average humidity of ~79%. Raw milk was immediately cooled to 4°C and maintained at this temperature during transport to the laboratory for analysis. Total bacterial counts for each raw milk sample were below 300,000 cfu/mL, and the SCC were below 200,000 cells/mL. Raw milk samples were stored at 1 to 4°C for 2, 24, 48, and 72 h before freezing at −20°C (Table 1: R_2, R_{24}, R_{48}, and R_{72}, respectively). The average fat content of the raw buffalo milk samples was 6.87 ± 0.62 g/100 g of milk; thus, HTST pasteurization was performed at 72°C for 1 5s. Following pasteurization, the milk samples were immediately cooled to 4°C. The pasteurized milk samples were then stored at 1 to 4°C for 7, 14, or 21 d (Table 1: P_7, P_{14}, and P_{21}, respectively) before freezing at −20°C. Time points consisted of 3 separate milk sets representing each of the 3 dairy herds sampled; thus, a total of 21 DNA samples were obtained and analyzed. Prior to DNA extraction all samples were stored at −20°C and then defrosted at 4°C.

Extraction of DNA

Bacteria obtained from 10-mL milk samples were collected by centrifugation at 13,200 × *g* for 15 min at 4°C. Fat and cell-free supernatants were removed, and cells were washed with 10-mL sterilized ultrapure water and centrifuged again at 13,200 × *g* for 5 min at 4°C to remove excess fat. Cells were resuspended in 2 mL of PBS, and 1.5 mL of the suspension was used for the preparation of total genomic DNA using the PowerFood Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. An additional 10-min incubation step at 75°C was incorporated before step 5 of the kit, as described previously (Quigley et al., 2012). The purity and integrity of the extracted DNA was determined by spectrophotometric analysis with a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis, respectively.

High-Throughput Sequencing and Bioinformatics Analysis

The PCR amplifications were conducted with the 515f/806r primer set that amplifies the V4 region of the 16S rRNA gene (Caporaso et al., 2011). All PCR reactions were carried out in 30-μL reactions with 15 μL of Phusion High-Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA), 0.2 μ*M* of forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min; followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s, and a final extension at 72°C for 5 min. The reverse primer contained a 6-bp error-correcting barcode unique to each sample. The paired-end sequencing was performed on an Illumina Miseq platform (Novogene, Beijing, China) based on a standard protocol from the manufacturer. Sequences were analyzed with QIIME (Caporaso et al., 2010) software package and UPARSE pipeline (Edgar, 2013). Pairs of reads from the original DNA fragments were merged

Table 1. Time points when milk samples were collected for analysis of the indigenous microflora

Item	Refrigerated storage time
Raw milk[1] samples (h)	
R_2	2
R_{24}	24
R_{48}	48
R_{72}	72
Pasteurized milk[2] samples (d)	
P_7	7
P_{14}	14
P_{21}	21

[1]Raw milk samples were collected from 3 different farms and stored at 1 to 4°C for 2, 24, 48, and 72 h (R_2, R_{24}, R_{48}, and R_{72}, respectively).

[2]The R_2 samples from each farm were pasteurized at 72°C for 15 s, cooled to 4°C, and stored at 1 to 4°C for 7, 14, and 21 d, respectively (P_7, P_{14}, and P_{21}, respectively). In total, 21 DNA samples were obtained. All samples were analyzed by high-throughput sequencing.