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 ($\leq 4^\circ\text{C}$) 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 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

Received February 17, 2016.

Accepted May 24, 2016.

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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 ($\leq 4^{\circ}\text{C}$) 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 $\sim 79\%$. 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 15 s. 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 $13,200 \times g$ 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 $13,200 \times g$ 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 μM 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 using FLASH (Magoč and Salzberg, 2011), and filtered by QIIME quality filters. The percentage of tagged se-

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

¹Raw 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).

²The 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.

quences used for analysis is shown in Table 2 [Effective % = (E × 100)/R; where E = number of effective tags and R = total number of raw pair end reads]. Default settings for Illumina processing in QIIME were used and the UPARSE pipeline was used to generate operational taxonomic units (OTU). The Phred quality score (Q score) was used to evaluate the read quality; for each sample ≥96% of the reads exceeded a quality score of Q30 (error probability of <1/1,000; Table 2). Sequences were assigned to OTU at 97% similarity. A representative sequence was picked for each OTU. The RDP classifier (Wang et al., 2007) was used to assign each OTU to a taxonomic level. Bacterial clustering was analyzed at phylum and genus level, and to reveal alpha diversity QIIME was used to generate rarefaction curves, Shannon-Wiener and Chao1 diversity indices, and Good's coverage estimator values. Good's coverage [1 – (number of singleton OTU/total number of individuals)] was used to evaluate the sequencing depth. Sufficient sequencing depth is represented by a value near 1, and all samples analyzed in our study had Good's coverage values between 0.974 and 0.995 (Table 2). All data shown represents the mean (±SD) of sequencing results obtained from 3 individual milk samples at each time point (21 milk samples were analyzed in total). The 3 samples represent milk collected from each buffalo dairy herd used in the study.

Statistical Analysis

Data were analyzed by SPSS 17.0 software (IBM Corp., Armonk, NY), with multiple comparisons made using the Least Significant Difference (LSD) method at a 0.05 level.

RESULTS

Quality Control and Diversity Analysis

Rarefaction analysis revealed that the overall depth of sequencing was sufficient, and that sample diversity decreased during prolonged storage under refrigeration conditions (1–4°C), with the 2-h raw milk (R₂) and 7-d pasteurized milk (P₇) samples displaying higher species diversities (Figure 1). Quality control, diversity, and richness estimations for each group are shown in Table 2. The number of clean reads increased significantly (*P* < 0.05) in raw milk samples during cold storage, from 49,079 (R₂) to 99,513 after 48 h (R₄₈). The Shannon and Chao1 diversity indices also decreased significantly during storage (*P* < 0.05), further confirming the presence of a less-diverse microflora. A transition to a less-diverse microflora was also observed in pasteurized milk samples where the number of clean reads increased after 21 d of cold storage (*P* < 0.05), and both the Shannon and Chao 1 diversity indices decreased by 14 d of storage (Table 2).

Bacterial Community Composition of Raw Milk

The microbial population of raw buffalo milk during storage at 1 to 4°C was investigated at both the phyla (Figure 2A) and genus levels (Figure 2B). In addition, a more precise percentage of the bacterial composition present at more than 1% of the total population is reported in Table 3. After 2 h of storage at 4°C, the raw milk was dominated by bacteria within the *Firmicutes* and *Proteobacteria* phyla, with each comprising more than 40% of the total population (Table 3). Following 24

Table 2. High-throughput sequencing quality control and bacteria diversity indices

Index	R ₂	R ₂₄	R ₄₈	R ₇₂	P-value
Bacteria of raw milk ¹					
Clean reads	49,079 ± 99 ^c	55,225 ± 6,029 ^c	99,513 ± 27,851 ^b	179,519 ± 27,658 ^a	0.000
Effective (%)	91.41 ± 0.93 ^a	90.39 ± 2.05 ^a	90.40 ± 1.71 ^a	90.21 ± 1.49 ^a	0.722
Q30 (%)	97.34 ± 0.32 ^b	97.66 ± 0.14 ^{ab}	97.71 ± 0.08 ^a	97.70 ± 0.16 ^{ab}	0.137
Good's coverage	0.979 ± 0.005 ^b	0.990 ± 0.004 ^a	0.994 ± 0.001 ^a	0.995 ± 0.002 ^a	0.002
Shannon diversity	6.94 ± 1.09 ^a	4.76 ± 1.60 ^b	3.93 ± 0.28 ^b	3.67 ± 0.16 ^b	0.018
Chao1 diversity	2,133 ± 687 ^a	978 ± 326 ^b	531 ± 81 ^b	501 ± 195 ^b	0.003
	R ₂	P ₇	P ₁₄	P ₂₁	
Bacteria of pasteurized milk ²					
Clean reads	49,079 ± 99 ^b	49,379 ± 8,162 ^b	73,068 ± 9,558 ^b	155,508 ± 45,246 ^a	0.002
Effective (%)	91.41 ± 0.93 ^a	88.32 ± 0.80 ^b	89.42 ± 1.92 ^{ab}	90.74 ± 1.74 ^{ab}	0.074
Q30 (%)	97.34 ± 0.32 ^{ab}	96.99 ± 0.48 ^b	97.65 ± 0.03 ^a	97.64 ± 0.10 ^a	0.068
Good's coverage	0.979 ± 0.005 ^{bc}	0.974 ± 0.008 ^c	0.986 ± 0.003 ^{ab}	0.995 ± 0.003 ^a	0.006
Shannon diversity	6.94 ± 1.09 ^a	7.51 ± 1.29 ^a	4.36 ± 0.69 ^b	1.95 ± 0.11 ^c	0.000
Chao1 diversity	2,133 ± 687 ^{ab}	2,586 ± 767 ^a	1,294 ± 313 ^{bc}	500 ± 221 ^c	0.007

^{a-c}Mean values with a different superscript differ by *P* < 0.05.
¹Mean of raw milk samples from 3 different farms stored at 4°C for 2, 24, 48, or 72 h (R₂, R₂₄, R₄₈, and R₇₂, respectively).
²Pasteurized milk samples were stored at 4°C for 7, 14, and 21 d (P₇, P₁₄, and P₂₁, respectively).

h of storage, the number of *Firmicutes* increased significantly ($P < 0.05$), but then their numbers dropped after 72 h of storage to levels similar to the 2-h sample (R_2 ; Figure 2A; Table 3). The abundance of *Proteobacteria* significantly decreased ($P < 0.05$) following 24 h of cold storage, but then recovered and was observed to be the dominant phyla in raw milk stored for 72 h (Figure 2A; Table 3). The *Bacteroidetes* population within raw milk remained constant throughout the study, whereas the *Actinobacteria* population appeared to decline gradually, with the population difference observed between the R_2 and R_{72} samples being statistically significant (Table 3). At the genus level, the 3 dominant populations after 2 h were *Streptococcus* (14.20%), *Lactococcus* (8.20%), and *Pseudomonas* (4.93%). After 24 h, *Lactococcus* and *Streptococcus* populations increased significantly ($P < 0.05$), with the *Lactococcus* population comprising 38.6% of the total microflora. The percentage of lactococci and streptococci decreased as the milk was stored for 48 and 72 h, with their respective percentages at 72 h being similar to 2 h of storage (Table 3). Prolonged storage at refrigeration temperatures resulted in a significant increase in *Pseudomonas* ($P < 0.05$), which represented the dominant genus after 72 h (Figure 2B) of storage, accounting for more than 51% of total bacteria (Table 3). In addition, the presence of bacteria within the *Acinetobacter* genus increased from 1.4 (R_2) to 11.2% (R_{72}) during the course of the study, meaning the total proportion of *Pseudomonas* and *Acinetobacter* was 62% after 72 h of storage (Table 3).

Figure 1. Rarefaction analysis at the 97% sequence similarity level. Raw and pasteurized milk samples were stored at 4°C for various times before rarefaction analysis: R_2 = raw milk samples stored for 2 h, R_{24} = raw milk samples stored for 24 h, R_{48} = raw milk samples stored for 48 h, R_{72} = raw milk samples stored for 72 h, P_7 = pasteurized milk samples stored for 7 d, P_{14} = pasteurized milk samples stored for 14 d, P_{21} = pasteurized milk samples stored for 21 d. OTU = operational taxonomic units.

High-Throughput Sequencing of Pasteurized Milk

Metagenomic analysis of the indigenous microflora in pasteurized buffalo milk was performed at the phyla (Figure 3A) and genus levels (Figure 3B). The percent change for bacteria present at higher than 1% of the total population is shown in Table 4. Although we found no significant population changes when looking at bacterial phyla in pasteurized milk stored for 7 d, analysis at the genus level showed a statistically significant increase in the percentage of lactococci and decrease in the percentage of streptococci at this time point (P_7 ; Table 4). *Firmicutes* and *Proteobacteria* were the dominant phyla present at 7 d of storage, representing more than 90% of the total population (Table 4); however, by 14 d of storage the percentage of *Proteobacteria* began to decrease and the number of *Firmicutes* significantly increased (Figure 3A; Table 4). After 21 d of storage it was observed that the *Firmicutes* were the dominant phyla at more than 90% of the total population (Table 4); this coincided with a significant increase in the number of *Paenibacillus* present, which reached more than 80% of the total microbial composition (Figure 3B; Table 4). The percentages of other bacterial genera shown in Table 4 decreased after 21 d of cold storage, with the exception of *Acinetobacter*, which remained constant at approximately 1% of the total population (Figure 3B).

DISCUSSION

In our study, 16S rRNA profiling provided detailed insights into the complex microbiota of raw and pasteurized buffalo milk. Our results showed that both raw and pasteurized milk contained a diverse microbial population, which underwent significant changes during prolonged storage at refrigeration temperature. Diversity index analysis indicated that the population within both raw and pasteurized milk gradually decreased with longer storage times, with the raw milk stored for 2 h (R_2) and pasteurized milk stored for 7 d (P_7) containing the most diverse microbial populations. In both milks, the populations became significantly less diverse over time, presumably due to the growth of psychrotrophic bacteria. However, the dominant bacterial phyla differed between milk samples at the final time point analyzed, with *Proteobacteria* comprising more than 60% of the raw milk population and *Firmicutes* making up more than 90% of the pasteurized milk microflora.

A previous study showed that the main bacteria phyla present in raw cow milk were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* as detected by 16S rRNA sequencing (Zhang et al., 2015). A separate study found similar results with these phyla making up

Figure 2. Composition of the indigenous microflora, at the phyla (A) and genus (B) levels, in raw milk samples stored under refrigerated temperatures. Data represent the mean percentage from the metagenomics analysis of 3 separate raw milk samples. R₂ = raw milk samples stored for 2 h, R₂₄ = raw milk samples stored for 24 h, R₄₈ = raw milk samples stored for 48 h, R₇₂ = raw milk samples stored for 72 h.

58, 22, 17, and 4%, respectively, of the total microflora in bovine milk (Delbès et al., 2007). Results from our study identified the same 4 bacterial phyla dominating the microflora of raw buffalo milk; however, the relative abundance of *Proteobacteria* and *Firmicutes* were similar in buffalo milk at ~43%, and the amount of *Actinobacteria* was significantly lower at 5% of the total population.

Culturing the indigenous microflora of raw ovine milk suggested that lactococci comprise a dominant population in fresh milk, including being more prevalent than *Pseudomonas* (Sanjuan et al., 2003). Similarly, raw bovine milk contained a more diverse microbiota when fresh, with lactococci and streptococci being the dominant genera (Raats et al., 2011). When stored for more than 54 h in a dairy plant tank at refrigerated temperature the microbial population shifted, with *Pseudomonas* and *Acinetobacter* becoming more dominant. This agreed with the findings of this study reported in Table 3, where streptococci and lactococci dominated the bacterial composition in raw buffalo milk stored for up to 24 h. Prolonged storage resulted in a population shift in which psychrotolerant microorganisms, specifically *Pseudomonas* and *Acinetobacter*, became the predominant microorganisms (Table 3). The presence of high numbers of psychrotrophic bacteria has been considered the most common cause of milk spoilage (Sorhaug and Stepaniak, 1997; Hantsis-Zacharov and Halpern, 2007; Ercolini et al., 2009), with *Pseudomonas* reported to cause milk fat rancidity, color changes, and a bitter flavor (Ranieri et al., 2009; DeJonghe et al., 2011).

Whereas bacteria in the *Pseudomonas* and *Acinetobacter* genera dominated the microflora in cold-stored raw milk, their populations were not observed to in-

crease in pasteurized buffalo milk stored for up to 21 d (Table 4). It is presumed that their lack of growth was due to their sensitivity to pasteurization (Eneroth et al., 2000; FDA, 2011). As *Pseudomonas* population in raw buffalo milk significantly increased between 24 and 48 h, it appears that optimal processing conditions would include pasteurization of the milk within 24 h of collection to prevent the overgrowth of *Pseudomonas* and *Acinetobacter* psychrotrophs. Although pasteurization after 24 h may kill these bacteria, their increased levels could allow for the release of heat-resistant lipases and proteases from those bacteria, which have also been reported to reduce milk quality and shelf-life (Quigley et al., 2013a).

In our study, both raw and pasteurized buffalo milk samples were found to be free of bacteria within the *Bacillus* genus; however, ~2% of microflora in high-quality raw milk stored for 2 h (R₂) consisted of *Paenibacillus*. *Paenibacillus* was shown to be the predominant genus of bacteria present pasteurized buffalo milk after 14 and 21 d of storage, reaching 25 and 85% of the total microbial population, respectively. Previous studies have reported the presence of both *Paenibacillus* and *Bacillus* spp. in raw milk entering dairy processing plants (Huck et al., 2007) and that members of these genera were the dominant gram-positive spore-forming bacteria isolated from pasteurized cow milk (Huck et al., 2008). Control of *Bacillus* and *Paenibacillus* spp. is important to the dairy industry, as their endospores can survive HTST pasteurization, allowing them to persist and potentially cause milk spoilage (Huck et al., 2008). Previous work has shown that most *Paenibacillus* isolated from pasteurized milk have the ability to grow at low temperatures, whereas only a few *Bacillus* typically grow under similar conditions (Ivy

Table 3. Influence of refrigerated storage time on the change in microflora (%) at both the phyla and genus levels in raw milk where the overall mean was greater than 1% of total bacterial composition¹

Item	R ₂	R ₂₄	R ₄₈	R ₇₂	P-value
Phylum					
<i>Firmicutes</i>	42.50 ± 9.68 ^c	80.36 ± 5.15 ^a	57.30 ± 7.69 ^b	32.83 ± 5.24 ^c	0.000
<i>Proteobacteria</i>	43.07 ± 16.50 ^b	14.00 ± 3.94 ^c	35.13 ± 4.95 ^b	64.60 ± 6.16 ^a	0.001
<i>Actinobacteria</i>	5.30 ± 3.11 ^a	2.40 ± 1.85 ^{ab}	3.20 ± 2.00 ^{ab}	0.70 ± 0.30 ^b	0.130
<i>Bacteroidetes</i>	2.87 ± 2.11 ^a	2.07 ± 1.64 ^a	1.83 ± 0.71 ^a	1.13 ± 1.29 ^a	0.601
Genus					
<i>Lactococcus</i>	8.20 ± 2.27 ^c	38.60 ± 7.02 ^a	22.50 ± 6.76 ^b	11.17 ± 2.47 ^c	0.000
<i>Streptococcus</i>	14.20 ± 3.16 ^{ab}	16.73 ± 4.30 ^a	14.63 ± 4.20 ^{ab}	8.57 ± 2.39 ^b	0.107
<i>Paenibacillus</i>	2.13 ± 0.68 ^b	2.33 ± 0.25 ^b	4.30 ± 1.77 ^a	2.43 ± 0.75 ^{ab}	0.102
<i>Pseudomonas</i>	4.93 ± 0.81 ^c	3.63 ± 1.30 ^c	17.20 ± 4.53 ^b	51.00 ± 1.87 ^a	0.000
<i>Acinetobacter</i>	1.40 ± 0.36 ^b	2.27 ± 0.57 ^b	3.60 ± 1.56 ^b	11.23 ± 4.25 ^a	0.003
<i>Enhydrobacter</i>	2.30 ± 0.26 ^{bc}	4.37 ± 1.80 ^{ab}	5.20 ± 1.28 ^a	1.80 ± 0.52 ^c	0.019
<i>Corynebacterium</i>	3.23 ± 0.75 ^a	2.00 ± 0.52 ^b	1.10 ± 0.56 ^{bc}	0.20 ± 0.17 ^c	0.001

^{a-c}Mean values within a row that have a different superscript differ by $P < 0.05$.

¹Mean percentages were determined from the metagenomic analysis of 3 separate raw milk samples at each time point (R₂: 2 h; R₂₄: 24 h; R₄₈: 48 h; R₇₂: 72 h).

Figure 3. Composition of the indigenous microflora, at both the phyla (A) and genus (B) levels, in pasteurized milk stored at refrigerated temperature. Data represent the mean percentages from the metagenomic analysis of 3 separate raw milk samples. R₂ = raw milk samples stored for 2 h, P₇ = pasteurized milk samples stored for 7 d, P₁₄ = pasteurized milk samples stored for 14 d, P₂₁ = pasteurized milk samples stored for 21 d.

et al., 2012). Hence, *Bacillus* spp. have been shown to represent a predominant population in the early shelf-life of pasteurized milk (7 d), with *Paenibacillus* spp. becoming the predominant bacteria, representing over 95% of the population during longer storage times (10 d; Ranieri and Boor, 2010). In fact, it has been reported that PCR-based testing of raw milk for the presence of *Paenibacillus* spp. may assist in predicting the shelf-life performance of pasteurized fluid milk products (Ranieri et al., 2012). Our results identified *Paenibacillus* in the raw milk collected for transport and showed it was the predominant bacterial genus found in pasteurized buffalo milk stored for 21 d. This suggests they may play a significant role in the spoilage of pasteurized buffalo milk. Although they represented the predominant bacterial genus in stored pasteurized milk, the population of *Paenibacillus* did not increase during the storage of raw buffalo milk, suggesting that other constituents of the raw milk population prevented their outgrowth. As seen with bovine milk, the results from our study suggest that technologies need to be developed for eliminating gram-positive spore-formers from raw buffalo milk to extend the shelf life of dairy products produced using pasteurized buffalo milk.

A significant population of lactococci and streptococci was identified within the raw milk microflora after 2 (R_2) and 24 h (R_{24}) of storage. The presence of lactic acid bacteria with raw milk is well established, and they have been investigated for their potential to improve human or animal health by the production of natural antimicrobials or other probiotic-associated traits (i.e., antihypertensive activity; Quigley et al., 2013b). More recently, the isolation of potential probiotic bacteria has been described from dairy foods produced with water buffalo milk (Jeronymo-Ceneviva et al., 2014), sug-

gesting that the raw milk from Chinese water buffalo may be a source for novel probiotic bacteria. Further studies should look at the potential for isolating these beneficial bacteria from raw buffalo milk that has been stored for no more than 24 h when the population of lactic acid bacteria appears abundant (Table 3).

CONCLUSIONS

This report describes the diverse bacterial community found in raw and pasteurized buffalo milk from Guang-Xi, China. The composition for the microbial community at the phylum and genus levels was characterized in both raw and pasteurized milk samples by high-throughput sequencing to demonstrate the population shifts which occur during long-term storage at refrigeration temperature ($\leq 4^\circ\text{C}$). Results showed that bacteria within the *Lactococcus* and *Streptococcus* genera dominated the raw milk microflora when stored for up to 24 h, but extended storage resulted in an outgrowth of *Pseudomonas* and *Acinetobacter*, which comprised more than 60% of the total population at 72 h. In pasteurized milk, *Paenibacillus* dominated the microbial population after 21 d of storage. Although the population changes differed between raw and pasteurized milks, the predominant bacterial populations identified after prolonged storage were psychrotrophic microorganisms commonly associated with the spoilage of dairy products. Whereas pasteurization appeared sufficient for eliminating contaminants from the *Pseudomonas* and *Acinetobacter* genera, this study demonstrated the need for developing novel methods or technologies for controlling the proliferation of *Paenibacillus* to extend the shelf-life of pasteurized buffalo milk products.

Table 4. Influence of refrigerated storage time on the change in microflora (%) at both the phyla and genus levels in pasteurized milk, where the overall mean was greater than 1% of the total bacterial composition¹

Item	R_2	P_7	P_{14}	P_{21}	<i>P</i> -value
Phylum level					
<i>Firmicutes</i>	42.50 $\pm$ 9.68 ^c	47.70 $\pm$ 5.70 ^c	68.26 $\pm$ 10.21 ^b	93.67 $\pm$ 3.50 ^a	0.000
<i>Proteobacteria</i>	43.07 $\pm$ 16.50 ^a	42.53 $\pm$ 2.57 ^a	26.33 $\pm$ 11.30 ^a	5.67 $\pm$ 2.87 ^b	0.006
<i>Actinobacteria</i>	5.30 $\pm$ 3.11 ^a	4.73 $\pm$ 0.90 ^a	1.17 $\pm$ 0.15 ^b	0.27 $\pm$ 0.12 ^b	0.011
<i>Bacteroidetes</i>	2.87 $\pm$ 2.11 ^a	1.27 $\pm$ 0.37 ^{ab}	0.80 $\pm$ 0.36 ^b	0.10 $\pm$ 0.10 ^b	0.070
Genus level					
<i>Lactococcus</i>	8.20 $\pm$ 2.27 ^b	18.53 $\pm$ 1.95 ^a	17.23 $\pm$ 2.97 ^a	3.03 $\pm$ 1.03 ^c	0.000
<i>Streptococcus</i>	14.20 $\pm$ 3.16 ^a	7.33 $\pm$ 1.38 ^b	9.23 $\pm$ 2.85 ^b	2.30 $\pm$ 0.35 ^c	0.001
<i>Paenibacillus</i>	2.13 $\pm$ 0.68 ^c	3.43 $\pm$ 0.93 ^c	25.37 $\pm$ 5.30 ^b	84.77 $\pm$ 4.75 ^a	0.000
<i>Pseudomonas</i>	4.93 $\pm$ 0.81 ^a	5.70 $\pm$ 0.75 ^a	5.47 $\pm$ 0.64 ^a	2.00 $\pm$ 0.53 ^b	0.001
<i>Acinetobacter</i>	1.40 $\pm$ 0.36 ^a	1.63 $\pm$ 0.67 ^a	1.83 $\pm$ 0.40 ^a	1.17 $\pm$ 1.24 ^a	0.732
<i>Enhydrobacter</i>	2.30 $\pm$ 0.26 ^a	1.93 $\pm$ 0.40 ^a	2.87 $\pm$ 1.14 ^a	0.17 $\pm$ 0.06 ^b	0.004
<i>Corynebacterium</i>	3.23 $\pm$ 0.75 ^a	2.40 $\pm$ 0.72 ^a	0.50 $\pm$ 0.26 ^b	0.10 $\pm$ 0.00 ^b	0.000

^{a-c}Mean values within a row that have a different superscript differ by $P < 0.05$.

¹Mean percentages were determined from the metagenomic analysis of 3 separate pasteurized milk samples at each time point (P_7 : 7 d; P_{14} : 14 d; P_{21} : 21 d of storage). R_2 : raw milk samples stored for 2 h.

ACKNOWLEDGMENTS

Financial support for this study was through the state science and technology support program of the 12th five-year plan (2013BAD18B12-3) and basic research funds of Guangxi Buffalo Research Institute (1404006; Guangxi, P. R. China).

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