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High-Throughput Sequencing for the Detection of the Bacterial Diversity in Milking Procedure in China Dairy Farms

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Abstract

The effect of proper milking procedure on improving raw milk of dairy farms was significantly. To explore the bacterial community along milking, 6 typical sites of milking before sending to the milk plant were selected in Hebei conventional dairy farm, including pre-sterilized cow's teats (C1), post-sterilized cow's teats (C2), milking cluster (E1), milk storage equipment (E2) and the different links of raw milk samples, milk in storage tank (M1) and milk in the transporters (M2). High-throughput sequencing technology has been used to study the characteristics of the bacterial diversity, richness and alpha diversity, beta diversity along milking. A total of 1 969 296 raw reads and 1 763 746 quality control sequences were obtained which were clustered into 3 546 OTUs. These OTUs were covered 33 phyla, 80 classes, 129 orders, 226 families, 457 genera and 213 species. The relative content of each milking sites is more than 5% dominant bacterium phyla: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria*; Dominant bacteria genera include *Acinetobacter, Arthrobacter, Sphingobacterium, Macrococcus and Corynebacterium*. Through analyzing bacterial OTUs and diversity, detected that composition of bacterial communities were clearly different at different milking sties, C1 has the highest bacterial richness and M1 has the best bacterial evenness. The diversity of bacteria was different and the richness and evenness were obviously different. The purpose of this study was to addresses the bacteria sources along milking, which can guide the raw milk utilization and production by consumers and dairy industry.

Keywords: Conventional dairy farms • Milking procedure • 16S rRNA • Bacterial community • Diversity • Differences

Introduction

Milk is nature's most complete food and considered to be the most nutritious food which contains a large number of essential nutrients and micronutrients, such as proteins, lipids, carbohydrates, vitamins and enzymes [1,2]. Many of these compounds have been proven to have beneficial effects on human nutrition and health [3-5]. Because of its nutritional properties, milk is also a good growth matrix for a variety of spoilage and potentially pathogenic microorganisms which harmful to human health. With the rapid development of CHINA economy, the increasing demand for dairy products, milk safety problems become the focus of social attention. Since the "melamine" milk powder incident in 2008, the annual growth rate of China's dairy output is less than 2%, showing a typical "hill-climbing peak" Phenomenon. This problem is mainly caused by consumers lack of confidence in China's dairy products [6]. Therefore, how to increase consumers' confidence in domestic milk and ensure the quality and safety of milk is the key to the development of China's dairy industry. Experts suggest that through the implementation of high-quality milk project, make consumers feel free to consume high-quality dairy products, which needs to start with raw milk, the first to ensure the quality and safety of raw milk.

The quality of milk is affected by many factors: health status of cows, milk handling and hygiene of milking. As the most upstream of the dairy supply chain, the quality and safety of raw milk are the main factors restricting the sustainable and healthy development of the dairy industry. The harmful microorganisms in raw milk are mainly bacteria, its spoilage causes significant economic losses for the food industry also can affect the health of consumers and even lead to death while lowering the quality of milk. A growing number of scientific studies indicated that the contamination of raw milk before milking was very low, mainly during milking and milk storage [7]. The raw milk secreted by healthy cows is in a relatively sterile state, but the raw milk is inevitably contaminated by microorganisms at every link from being squeezed out to being transported to dairy processing enterprises [8]. In conventional milking parlor, the quality of raw milk produced by healthy lactating cows is affected by a number of ways in milking procedure, such as the sanitary condition of milking parlors, the preparation of milking in the early stage, the cleanliness of the teats, the milk treatment procedures and the cleanliness of milking cluster and storage equipment, etc. Previous studies have found that milking procedure is an important factor affecting the total number of bacterial colonies in raw milk [9]. Proper milking procedure in dairy farms is an important factor affecting the quality and safety of raw milk. In China, the research focuses on the research of pathogenic bacteria but

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there is little research on the microorganism pollution source of raw milk [10,11]. In order to control the microbial contamination levels and milk safety risk in raw milk, bacteria community of milking links in dairy farms in china should be strictly regulated and controlled. Therefore, it is important to research bacterial community and its risk factor in raw milk in china.

In this study, 6 typical sites of milking in dairy farm in Hebei province of China were selected, including pre-sterilized cow's teats (C1), post-sterilized cow's teats (C2), milking cluster (E1), milk storage equipment (E2) and the different links of raw milk samples, milk of storage tank (M1) and milk in the transporters (M2). The bacterial community structure and diversity of the above 6 groups were studied by high-throughput sequencing. The results of this study can be used to predict the possible microbial species in raw milk, it provides the basis for the control of the source of microbial contamination in the raw milk and further standardizes and optimizes the milk productive process of the dairy farm to ensure the health of raw milk.

Materials and Methods

Description of different sampling sites of milking

Experimental dairy farm was located in Tangshan city, Hebei province, China. 6 control sites in the milking which have a key effect on the quality of raw milk were selected. 6 groups were: C1 (pre-sterilized cow's teats), C2 (post-sterilized cow's teats), E1 (milking cluster), M1 (milk in storage tank), E2 (milk storage equipment), M2 (milk in transport vehicle).

Sample collection

A total of 36 samples of 6 groups were collected from dairy farm. Collection of samples C1: 6 healthy cows were randomly selected, samples were taken with sterile cotton swab from the area of 1 cm^2 around the teats, and then placed in 10 mL sterile normal saline immediately; Collection of samples C2: Samples were taken with sterile cotton swab from the cows corresponds to C1 in the same way; Sample collection of E1: After wiping the surface with sterile swabs and placed in 10 mL sterile normal saline; Collection of M1) and M2: After mixing milk well, the liquid milk bucket was used to sample milk from the surface, the middle and the bottom of the 3 points then thoroughly mixed and evenly, respectively 15 mL was taken and divided into 6 sample collection tubes; Milk storage equipment (E2): after wiping with sterile cotton swab and placed in 10 mL sterile normal saline. 6 biological replicates were collected at each sampling site. All samples were stored in liquid N2 immediately after collection and made backup.

Library construction

DNA was extracted from 36 samples of milking procedure. The V3-V4 hypervariable region of 16S rRNA were amplified by PCR for barcoded pyrosequencing The 16S rRNA gene V3-V4 region of bacteria was amplified using the universal Forward:5'-ACTCCTACGGGAGGCAGCAGCAG-3' and reverse 5'-GGACTACHVGGGTWTCTAAT-3'. Agencourt AMPure XP magnetic beads were used to isolate the amplicons and then the library was constructed. Agilent 2100 Bioanalyzer was used to detect the range and concentration of fragments in the library [12].

Data analysis

High-quality data were obtained by remove the low-quality sequences, which can be used for subsequent analysis [13]; FLASH V1.2.11 software was used to assemble the paired sequences obtained by double-terminal sequencing into a sequence by overlapping relationship, and tag sequences with high variable region were obtained [14]; USEARCH V9.1 was used to cluster the splice effective sequences with 97% similarity, and then the OTU representative sequences were compared with the Greengene database by RDP Classifer V2.2 software, and the species annotation of OTU was carried out [15-17]; Based on the results of OTU and species annotation, species complexity analysis and inter-group species difference analysis were carried out.

Abundance analysis, rarefaction analysis and significance analysis of intergroup differences

The α-diversity values of the samples were calculated using the Mothur (v1.31.2) software, and the corresponding rarefaction curves were generated using the R (v3.1.1) software. PCA was conducted to compare similarities among samples using R and the corresponding rarefaction curves were generated using the R (V3.1.1). Heatmap analysis was performed based on the relative abundance of each taxon within a sample and cluster analysis was initiated at genus level, and all taxa with an abundance of less than 20% in a sample were grouped at others. Intergroup differences in alpha-diversity indices were presented as box plots. β-Diversity heatmaps were generated using a heatmap in the NMF package of the R (v3.1.1) software. Cluster analysis was performed using the QIIME (v1.80) software.

Results

Sequencing data statistics and OTUs composition analysis

A total of 1 969 296 original sequences and 1 763 746 quality control sequences were obtained from the 36 samples at 6 different sampling sites of milking procedure. After clustering the merged tags, 3 546 OTUs at 97% identify were obtained based on the 16S rRNA data. Among them, the OTUs in E1 sample was the most, reaching 2 497 OTUs, while the OTUs in E2 sample was the least, only 1 027 OTUs.

OTU abundance analysis

The result of OTUs identification, among the 36 samples in 6 groups, the common number of OTUs is 1 257, of which C1 group has 48 unique OUTs, C2 has 150 unique OTUs, E1 has 182 unique OUTs, M1 has 119 unique OTUs, E2 has 120 unique OTUs and M2 has 116 unique OTUs, accounting for 2.12%, 6.76%, 7.29%, 5.81%, 11.70% and 5.78% of the total OUTs respectively (Figure 1). In addition, the results also showed that E1 (milking equipment) group has the most unique bacterial communities among 6 groups, indicated that E1 was the key link affecting the quality of raw milk.

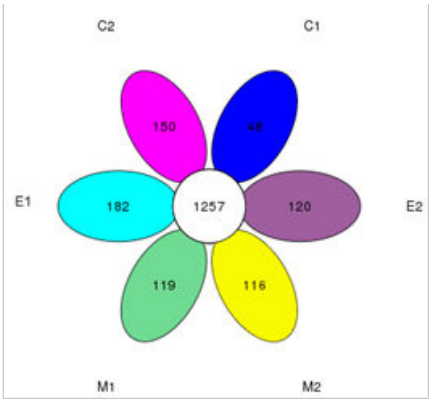

Figure 1. The picture of OTU Core-Pan of different sampling sites

Diversity and composition of bacterial communities

The Shannon index, Simpson diversity index, Chao1, ACE and Observed species of each sample were used to evaluate the species richness and diversity (Table 1), 5 indexes showed significant differences ($P<0.05$, respectively) and indicated there was a large distinction of species richness

and diversity of the bacterial community existed between 6 groups. The Simpson index for E2 group was higher than other 5 groups, and the Sobs index, Chao1 index, and Shannon index for E2 group were significantly lower than the other 5 groups, indicates the bacterial community of E2 group has a high richness. The Shannon index for E1 and M1 was significantly higher than other 4 groups, indicating that the bacterial community of E1 and M1 had a higher bacterial diversity and evenness. Meanwhile, Chao1 index also showed that the bacterial community of E1 and C1 had a high richness.

Table 1. Alpha diversity in different groups.

Sample/Info	Sobs index	Chao index	Ace index	Shannon index	Simpson index	Coverage
C1	1267.530 ± 171.852	1510.231 ± 201.751	1515.750 ± 205.170	4.827 ± 0.7345	0.041 ± 0.043	0.993 ± 0.002
C2	1250.333 ± 132.952	1360.722 ± 216.808	1361.686 ± 229.008	5.373 ± 0.297	0.017 ± 0.009	0.996 ± 0.003
E1	1401.333 ± 152.792	1489.633 ± 203.448	1480.794 ± 202.806	5.667 ± 0.063	0.012 ± 0.001	0.997 ± 0.002
M1	1002.667 ± 51.259	1018.735 ± 50.420	1011.785 ± 52.527	5.779 ± 0.092	0.008 ± 0.001	0.999 ± 0.000
E2	351.333 ± 159.439	383.601 ± 149.439	372.815 ± 151.463	2.567 ± 0.889	0.245 ± 0.167	0.999 ± 0.000
M2	978.566 ± 18.328	998.733 ± 33.006	986.595 ± 21.047	5.772 ± 0.0292	0.008 ± 0.001	0.999 ± 0.000

According to the sample number and species OTUs, we calculated the species accumulation curve of all participants, the curves of all samples had reached plateaus with the current sequencing, and the species had no more obvious increase as the sample number increased, which indicated that the sequencing depth and coverage was sufficient (Figure 2).

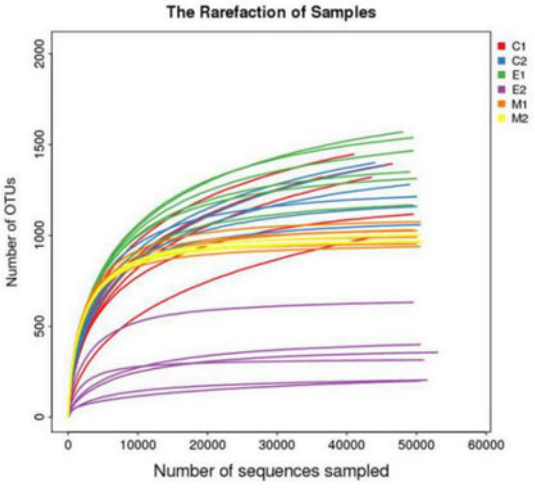

Figure 2. Rarefaction curves of the bacterial communities at different sampling sites.

Analysis of taxonomic annotations

Comparison of OTUs against the database at the phylum, class, order, family, genus, and species levels resulted in the annotation of the 16S rRNA sequence-based OTUs to 33 phyla, 80 classes, 129 orders, 226 families, 457 genus and 213 species.

Heatmap analysis

Heatmap clustering analysis were performed at the genus level. The top 10 most abundant bacterial species, based on the 16S rRNA sequences, were in descending order of *Acinetobacter, Kocuria, Arthrobacter, Sphingobacterium, Macrococcus, Corynebacterium, Knoellia, Chryseobacterium, Enhydrobacter, Microbacterium, Prevotella, Psychrobacter, minobacterTreponema*. To accurately evaluated the bacterial community composition at the genus level, heat map analysis of the top 28 genera was performed (Figure 3). Heatmap cluster analysis was initiated at genus level, and all taxa with an abundance of less than 20% in a sample were group at others.

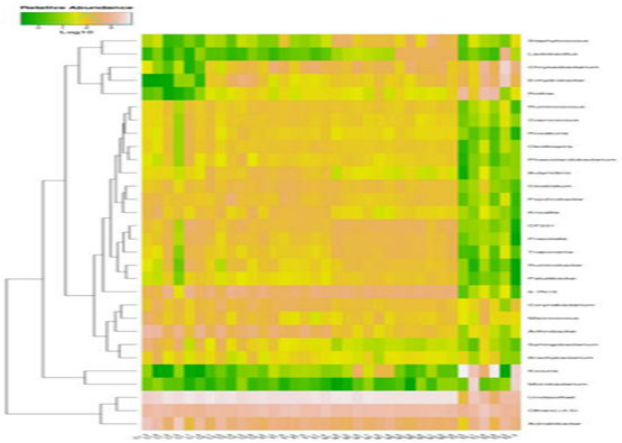

Figure 3. The heatmap in relative abundances of Top 28 abundant bacterial genera in milking procedure. C1 denotes the pre-sterilized cow's teats ; C2 denotes pre-sterilized cow's teats ; E1 denotes the milking cluster; M1denotes the milk in storage tank; E2 denotes the milk storage equipment; M2 denotes the milk in transport vehicle.

Significance analysis of intergroup differences

The NGS method was used for comparative analysis with Greengene database. Approximately 457 genera were detected. The relative abundances of 28 dominant bacterial genera were shown in Figure 4. In 6 groups the *Acinetobacter* is the dominant bacteria genera, which content in each group was more than 3.5%, accounted for 13.06% (C1), 6.31% (C2), 5.84% (E1), 6.96% (E2), 5.04% (M1), 3.90% (M2) at each sampling point, respectively. Besides, in C1 group, the dominant bacterial genera were *Acinetobacter, Arthrobacter* and *Sphingobacterium*.

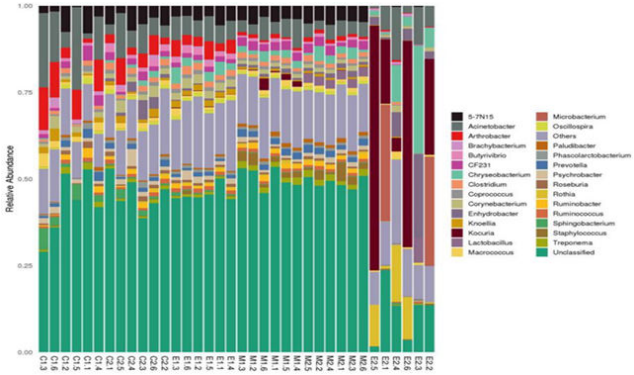

Figure 4. Biodiversity of 28 dominant bacterial genera expressed as relative abundance (%) of OTUs in milking procedure. The x-coordinate is the sample name, and the y-coordinate is the relative abundance of the species annotated. The classification level was not annotated were grouped at Unclassified and with an abundance of less than 20% in a sample were group at others. C1 denotes pre-sterilized cow's teats; C2 denotes the pre-sterilized cow's teats; E1 denotes the milking cluster; M1 denotes the milk in storage tank; E2 denotes the milk storage equipment; M2 denotes the milk in transport vehicle.

In C2 group, the dominant bacterial genera were *butyrivibrio* and *psychrobacter*; In E1 group, the dominant bacterial genera were *Clostridium, Corynebacterium, Knoellia* and *Oscillospira*; In E2 group, the dominant bacterial genera were *Kocuria, Chryseobacterium* and *Enhydrobacter*; In M1 group, the dominant bacterial genera were *Prevotella* and *Ruminobacter*; However, the dominant bacterial communities were changed in M2 group, consisting of *Lactobacillus, Lactococcus, Staphylococcus* and *Treponema*. The results indicated that bacterial community composition were different in different sampling sites of milking procedure.

Cluster analysis of species compositions in different samples

Cluster analysis showed that the bacterial community compositions of the M1 and M2 were quite similar, the bacterial community compositions of the C1, C2 and E1were quite similar, but E2 differs in species composition from the other 5 groups (Figure 5).

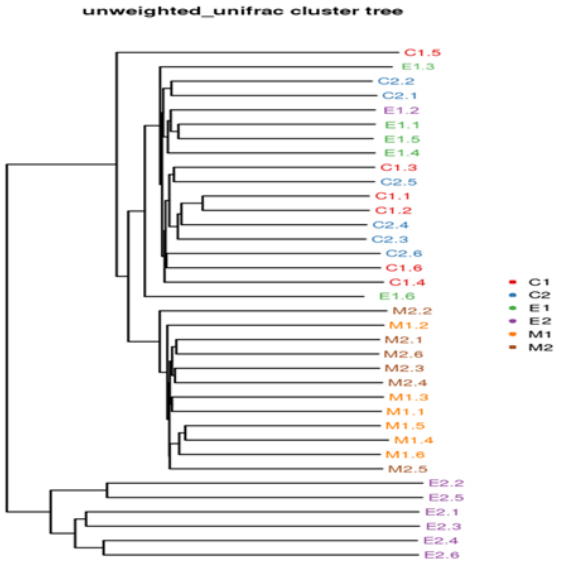

Figure 5. samples clustering result (Description, weighted_unifrac). The same color represents the samples in the same group. Short distance between samples represents high similarity. C1 denotes the pre-sterilized cow's teats; C2 denotes the pre-sterilized cow's teats; E1denotes the milking equipment; M1denotes the milk in storage tank; E2 denotes the (milk storage equipment; M2 denotes the milk in transport vehicle.

Significance analysis of intergroup differences

Principal Components Analysis (PCA) was performed based on the OTUs abundance. The bacterial community in M1 and M2 were relatively similar, two specimens of the raw milk are very close in the figure, and some points almost overlapped. In addition, the bacterial community in C1, C2 and E1 were relatively similar, it revealed that much of variance in bacterial communities of above 3 groups was associated with cleanliness of cow teats, milking cluster. However, there were significant differences between the E2 and other 5 groups in the bacterial community compositions of milking (Figure 6). The bacterial community structure of the milking sites showed an obvious clustering phenomenon, with most of them clustered to the left and only a few to the right.

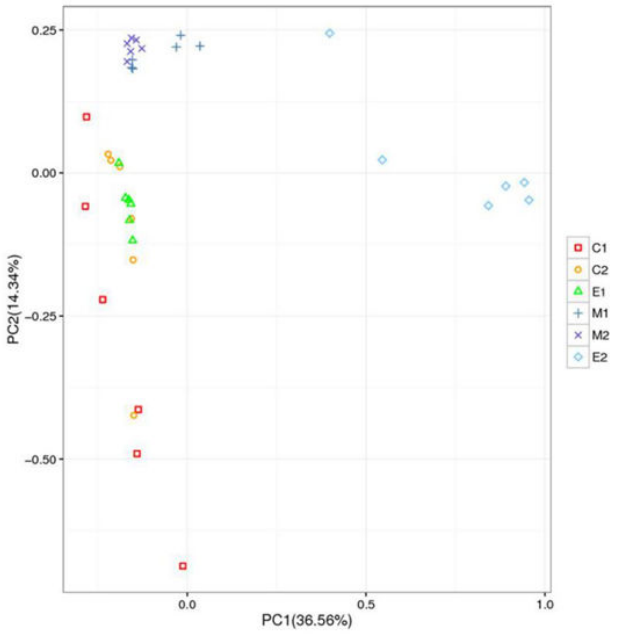

Figure 6. Principle components analysis based on operational taxonomic units abundance (description). X-axis, 1st principle component and Y-axis, 2nd principal component. Number in brackets represents contributions of principle components to differences among samples. Each small shape in the figure above represents a sample. The shapes of the same color are from the same group. The closer the distance between the two shapes is the smaller, the difference in community composition is. C1 denotes the pre-sterilized cow's teats; C2 denotes the pre-sterilized cow's teats; E1 denotes the milking cluster; M1 denotes the milk in storage tank; E2 denotes the milk storage equipment; M2 denotes the milk in transport vehicle.

Discussion

High-throughput next-generation sequencing, also known as "next generation" or "deep" sequencing, which can sequence hundreds of thousands to millions of DNA sequences in one time, so it is also called deep sequencing [18,19]. In recent years, high-throughput sequencing technology has been widely used in the study of dairy products, gradually changing from the identification of dominant flora to the studies on the overall diversity of microorganisms [20,21]. Milk was consumed worldwide and considered as relevant sources of nutrition in humans and animals, not only the newborns. Due to the complexity of the dairy chain, microbial contamination can occur in different steps of production, leading to the development of adequate control plans for monitoring the microbial quality and safety of milk since production to processing [22].

Milk in healthy udder cells is thought to be sterile [23] but there after becomes colonised by microorganisms from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil and other environments [23]. The milking procedure in conventional dairy farms is divided into several steps: observation of cow and equipment cleanliness, make sure there is no manure on the udder and teats; cow udder health inspection and make sure there is no mastitis and other disease; Dip the teats with an iodine or propylene glycol which can drastically reduce the incidences of infection [23]; Make the milking cluster properly adjusted to squarely hang under the udder and milking into the milk storage tank [5,6]. Important microbial groups were researched in different milking sites to assess the hygienic procedures and conditions during production, such as Mesophilic aerobes and Coliforms [22]; some groups are considered as relevant spoilage agents, such as *Sphingo bacterium, Pseudomonas* and *Clostridium*; Many bacteria are researched due to their pathogenic potential, such as *Acinetobacter, Arthrobacter, Staphylococcus, Campylobacter* and *Arcobacter*; and other bacteria can possess beneficial features, like some *Lactobacillus, Lactococcus, Streptococcus* and *Enterococcus* [23]. This huge

diversity is a challenge in the dairy industry, addresses their sources in different milking procedure, which can guide the raw milk utilization by consumers and dairy industry.

This study presented a novel investigation of the bacterial community in milking procedure. Udder health is an essential component of quality milk, mastitis is the common disease found in dairy herds in the China [24]. Cow teats surface can contain a high diversity bacteria, this study revealed that *Acinetobacter* (13.06%) and *Arthrobacter* (7.43%) were detected in C1 but *Acinetobacter* (6.31%) and *Arthrobacter* (4.02%) in C2, there is a significant decrease in bacterial richness. Notably, teats disinfection is very important before milking which can reduce the diversity and richness of bacteria community. Jones T found the two basic principles of mastitis control are first, elimination of existing infections and, secondly, prevention of new infections [25]. Milking cluster is an important input in modern dairy farms, which can directly ACTS on the udder of cows and directly touches the raw milk and generally considered the major sources of contamination of raw milk [5,6]. This study detected that in addition to the high content of *Acinetobacter* (5.84%) and *Arthrobacter* (3.65%), *Clostridium* (1.85%), *Corynebacterium* (2.36%), *Knoellia* (1.65%), *Oscillospira* (1.09%) were also the dominant genus in E1group. According to the results of Samples Clustering (Description, Weighted_Unifrac) and PCA, there was a notable clustering phenomenon toward the C1, C2 and E1 reveals the bacterial community composition of the C1, C2 and E1 were quite similar.

Our research found that *Prevotella* (1.85%) and *Ruminobacter* (1.27%) were the dominant genus in M1. However, *Lactobacillus* (2.62%), *Staphylococcus* (2.15%), *Lactococcus* (1.18%) and *Troponema* (1.70%) were the dominant genus in M2. Cluster analysis showed that the bacterial community composition of M1 and M2 were quite similar, this results were partly consistent with previous studies, *T. Hagi* believed that there were two main strains in the milk, Lactobacillus and Staphylococcus [26]. *C. Delbes* detected that the dominant bacteria in milk were Clostridium and Lactobacillus [27]. Previous study shows that the dominant bacteria detected in the commercial milk were Acinetobacter and Pseudomonas [28]. E. A. Roasolofo believed that the abundance of Gamma proteobacteria and bacillus would increase with the prolongation of refrigeration time, so the processing time of raw milk into commercially available milk should be shortened [29,30]. Milk storage equipment (E2) can contain a reservoir of bacteria, this study detected that *Kocuria* (30.04%), *Chryseobacterium* (8.69%) and *Enhydrobacter* (6.64%) were the dominant genus bacteria in E2. The bacterial community composition of E2 was differs from other 5 groups, the reason for this difference may be caused by the temperature of the milk storage equipment and the microorganisms in the environment.

Besides, a variety of pathogenic bacteria genera were identified in this study, such as *Acinetobacter, Arthrobacter, Sphingosinolium, Staphylococcus, Pseudomonas* and *Corynebacterium*. *Acinetobacter* and *Corynebacterium* can cause bovine mastitis, Sphingomonas can decompose fat and protein in mlik result in reducing the quality of milk. *Bacillus anthracis* can produce enterotoxin, which is highly pathogenic to humans and animals [18,19]. *Acinetobacter* as a kind of conditional pathogenic bacteria, C1 group has the highest percentage (13.06%), followed by E2 (6.96%) and C2 (6.31%), the result suggests teats disinfection before milking is crucial and pathogenic bacteria of messy environment in the dairy farms will through the injured cow nipple cause mastitis. Bacterial community composition in different sampling sites of milking was significantly different, therefore, we believe that there is a considerable correlation between the proper milking procedure and raw milk quality. This study give a comprehensive and in-depth understanding of the bacterial diversity and composition along milking in dairy farms. It is of great significance to grasp the key nodes in the milk production process as a whole and provide a strong scientific basis for the quality and safety supervision of raw milk.

Conclusion

To sum up, the current study analyzed the bacterial community diversity along milking. Our results showed that clear structural differences existed in the microbiota of milking procedure. This study has provided interesting insights into the relationship between the bacterial community composition of raw milk and milking procedure. The results have also shown that pyrosequencing technique is useful for detecting a wide diversity of microorganisms along milking. The results obtained here will be valuable for screening for pathogenic bacteria from different milking procedures, which can guide us to conduct proper milking.

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Authors Contributions

Huihui Cao, Baiqin Zheng, Lixue Dong designed the study, Huihui Cao, Yanhua YAN analyzed the data and wrote the manuscript, Lei Wang, Yan Wang, Yu Zhou, Tang Xueying, Zhang Ning executed the study. All authors read and approved the final manuscript.

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