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16S rDNA High Throughput Sequencing Bacterial Diversity Analysis of Cow Mastitis

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ABSTRACT

Background: Bacterial mastitis in cows is a huge challenge to the dairy cattle breeding and production industry. This study examined the relationship between mastitis and bacterial diversity at different sampling sites in dairy farms.

Methods: High-throughput sequencing of 16S rRNA V4 region was used to analyze the abundance and diversity of bacterial population sampled from seven different sites in a dairy farm, named healthy cow milk (N), cow milk from a mastitis cow (C), perimammary (T), milk collecting cup (B), turntable surface (P), feces (F) and bedding material (W).

Result: In total, 2757356 reads were obtained from the sequencing results, which were annotated to 29 phyla, 67 classes, 166 orders, 295 families, 696 genera and 306 species. In all samples, Proteobacteria and Firmicutes were the dominant microflora, while Oscillospira-UCG-005 and Atopostipes were the dominant genera in different proportions, indicating certain differences in bacterial flora structure among different samples. Furthermore, alpha diversity analysis revealed high richness and diversity among different samples.

Key words: 16S rDNA, Bacterial diversity analysis, Cow mastitis, High throughput sequencing.

INTRODUCTION

The occurrence of cow mastitis has been associated with improper management of cattle farms or the special physiological status of cow mammary glands that allow the invasion of pathogenic microorganisms (Deng *et al.,* 2009). Mastitis in cows affects the milk production and quality of dairy products, which directly threatens the economic development of the dairy industry and human food safety (Wang *et al.,* 2015). Therefore, the prevention of dairy cow mastitis has become a serious issue. Cattle mattress is the most common source of pathogens infecting the udder. In absence of timely cleaning/disinfection, pathogenic bacteria rapidly multiply, especially in a hot and humid environment (Yazdankhah *et al.,* 2001). Proper disinfection of milking utensils and medicinal teat dip after milking can prevent mastitis in cows (Jones *et al.,*1985). Altogether, unhygienic conditions directly or indirectly cause mastitis in cows.

Presently, around 150 pathogenic microorganisms are reported to cause mastitis in cows, mainly including bacilli, cocci, mycoplasma, fungi, mycoplasma and viruses (Laventie *et al.,* 2019). The conditional pathogenic bacteria dominated by *Staphylococcus, Streptococcus* and *Escherichia coli* are responsible for >90% of the total incidence of cow mastitis (Wu *et al.*, 2017; Xia *et al.*, 2019). Therefore, only a comprehensive understanding of pathogens causing mastitis in different regions of cattle farms can help the management of mastitis disease. In this study, 16S rDNA amplicon sequencing technology was used to examine the composition and diversity of bacterial communities in different samples from a dairy farm, including healthy cow milk, cow milk from a mastitic cow and samples from the breast area, milk collecting cup, rotary table surface, fecal samples and bedding material to evaluate the animal health status and the safety of milk collection system and bedding materials. Our results can guide safe and reasonable breeding practices in cow farms.

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MATERIALS AND METHODS

A large-scale Holstein dairy farm in the Tangshan area of Hebei Province was the experimental site.

Milk samples

Six cows, including three healthy and three mastitis cows, were selected. Firstly, the cow udder was disinfected by washing with warm water, scrubbing with 0.2% bromogeramine (or iodine solution) and then wiping the teats with 75% alcohol cotton balls. The milk was manually squeezed wearing sterile gloves. The first three handfuls of milk in each milk chamber

were discarded to eliminate any possible bacterial contamination. Then, 5 ml of respective milk samples were collected in 15 ml sterile centrifuge tubes, which were marked with cow number, milk chamber date, *etc.*, before cryopreservation in liquid nitrogen. All samples had three replicates.

Samples from collecting cups and rotary table surfaces

After wiping the container surface or equipment with cotton swabs, samples were collected in Eppendorf tubes. A sterile wet cotton swab (in water) was applied within 10 m^2 of the test surface. The swab was immediately cut and placed into a test tube containing 10 ml of sterile water, which was stored in liquid nitrogen. Each sample had three replicates.

Samples from the udder area

Cows were smeared around the udder area to collect three samples from each cow. A sterile absorbent cotton ball was dipped in 0.9% normal saline and smeared on a selected 1 cm^2 area near the udder. The smeared cotton swabs were quickly put into 10 ml of normal saline and then stored in liquid nitrogen.

Fresh fecal samples

Peanut-sized feces from the cow rectum were collected in 1.5 ml tubes and the sample was stored in liquid nitrogen. All samples had three replicates.

Samples from the bedding material

The samples were collected from cow bedding at three different points. At each point, peanut-sized sand was taken into a 15ml sterile centrifuge tube, which was frozen in liquid nitrogen. All samples had three replicates.

All collected samples were sent to Beijing Nuohe Zhiyuan biological information technology Co., Ltd. for sequencing. Specific grouping information of the collected samples is shown in Table 1.

The diversity of bacterial community composition was analyzed by high-throughput sequencing. Total DNA was extracted from the sample following the instructions of the

Table 1: Statistics of microbial 16S rDNA data in different samples.

Sample ID	Raw data	Post-splicing sequence	Effective	Sequences after dataremoval of chimeras	Average length of final reads (nt)
F1	107,857	94,763	92,036	74,713	414
F2	115,984	100,847	98,139	76,695	414
F3	113,689	101,228	98,373	73,070	416
B1	90,622	66,484	64,609	50,541	406
B2	116,454	101,633	99,366	70,831	413
B3	111,492	101,221	98,427	68,503	416
P1	119,424	108,592	105,852	75,593	417
P2	74,299	62,265	60,694	46,168	410
P3	125,456	83,750	81,011	71,959	363
T1	119,071	102,344	99,926	72,810	412
T2	112,978	100,870	98,740	80,957	413
T3	74,459	58,998	57,531	45,957	410
N1.1	115,904	89,169	86,286	65,398	404
N1.2	103,837	76,527	73,973	57,687	406
N1.3	122,148	104,770	102,325	89,484	392
N2.1	113,281	64,067	61,511	55,135	378
N2.2	101,574	78,672	76,210	67,457	412
N2.3	106,831	81,982	79,577	67,041	418
N3.1	110,392	85,489	82,557	75,168	404
N3.2	117,917	70,166	67,004	60,300	388
N3.3	119,781	77,665	75,089	66,830	405
C1.2	100,849	65,751	63,287	51,348	395
C1.3	111,778	58,622	55,806	48,464	367
C2.1	100,612	78,855	75,892	65,412	421
C2.2	108,954	74,572	71,429	68,108	396
C2.3	117,789	90,810	87,527	81,345	418
C3.1	109,096	78,247	75,317	68,839	420
C3.2	115,628	114,419	112,054	104,551	419
C3.3	115,065	74,256	72,040	66,907	414
W1	107,500	93,506	90,677	80,132	422
W2	105,380	95,517	92,922	73,717	419
W3	122,401	121,299	119,526	104,753	417

bacterial genome Kit (E.Z.N.A. Stool DNA Kit, Omega) and the 16S rDNA gene was PCR amplified using the primers 515F (GTGCCAGCMGCCGCGGTAA) and 806 R (GGACTAC HVGGGTWTCTAAT). The amplified product was analyzed by 1% agarose gel electrophoresis and the band size of 400-450 bp was recovered by gel purification. The library was constructed with NEBNext® Ultra™ IIDNA Library Prep Kit (Cat No. E7645, New England Biolabs) and subjected to Qubit quantification and then sequenced on the Hiseq platform. All high-throughput sequencing-related work was completed by Beijing Nuohe Zhiyuan bioinformatics Co., Ltd.

The raw data were spliced and filtered to obtain clean data, followed by noise reduction using the DADA2 program. Sequences were filtered to obtain the final amplicon sequence variants (ASVs). Each representative ASV was annotated to obtain the corresponding species and species-based abundance distribution information. Meanwhile, the abundance, alpha diversity, Venn diagram and petal diagram of ASVs were analyzed to find out species richness and evenness within the sample and common and unique ASVs among different samples or groups. A phylogenetic tree from the multi-sequence alignment of ASVs was constructed to explore the differences in microbial community structure among different samples or groups. Dimension reduction analyses were performed by principal coordinates analysis (PCoA), principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) and the sample clustering tree was constructed.

T-test, MetaStat, LEfse and other statistical analyses were performed to find significant differences in species composition and community structure among different samples. The data are shown as mean±SD; Data with $p<0.05$ and <0.01 denote the significant and extremely significant differences, respectively.

RESULTS AND DISCUSSION

Assembly and splicing of microbial 16S rDNA sequences from different samples

Data obtained from 16S rDNA sequencing of 33 samples (healthy cow milk (N1, N2 and N3), cow milk from a mastitic cow (C1, C2 and C3) and samples from the udder area (T), milk collecting cup (B), rotary table surface (P), feces (F) and bedding material (W)) were analyzed after quality control. As shown in Table 1, the maximum numbers of valid data were from W3 (121299), followed by C3.2 (114419) and then P1 (108592); the total effective and average data volumes were 2757356 and 86167.375, respectively. The large sequencing numbers indicated data accuracy. The average length distribution range in 7 groups of samples after removing chimeras was 363-422 nt, which mainly concentrated between 404-420 bp. This was close to the designed primer length of 400-450 bp satisfying sequencing analysis.

Alpha diversity analysis of 7 groups of samples

According to the OTU data, the dilution and species accumulation curves are shown in Fig 1 and 2, respectively. The dilution curve of 32 samples tended to be flat after a sequencing depth of 10000-15000 indicating substantial diversity in microbial flora (Fig 1). The species accumulation curve reflected the emergence rate of new ASVs under continuous sampling. Within 1-30 samples, the species accumulation curve showed a sharp rise, indicating the presence of several bacterial species in the community. For the sample size of ~ 32, the curve tended to be flat, indicating saturation in the numbers of microbial species (Fig 2).

Bacterial composition analysis of 7 groups of samples

Based on the species classification analysis of OTU at 97% similarity, the bacterial species in 33 samples of 7 groups were annotated to 29 phyla, 67 classes, 166 orders, 295 families, 696 genera and 306 species. As shown in Table 3, the top 10 most abundant species were selected at the category level. In the 7 groups of samples, Proteobacteria and Firmicutes were the dominant microflora. The abundance of Proteobacteria was 8.52, 12.95, 6.99, 20.79, 13.58, 13.79 and 0.53% and the abundance of Firmicutes was 33.88, 7.82, 14.15, 11.56, 11.18, 12.30 and 0.30% in F, B, P, T, N, C and W samples, respectively. There were significant differences in microbial communities among the 7 groups of samples. The relative abundance of Chloroflexi in N was much lower than that in T, C and W samples (Table 2 and Fig 3).

Further analysis at the genus level revealed a relatively low abundance of many species. The top 30 most abundant genera were selected for subsequent analysis (Table 3 and Fig 4). *Oscillospira-ucg-005* and *Atoprostipes* dominated the microbial community in all 7 groups of samples. The abundance of *Oscillospira-UCG-005* in F, B, P, T, N, C and W samples was 14.25, 6.66, 9.91, 8.66, 1.82, 1.10 and

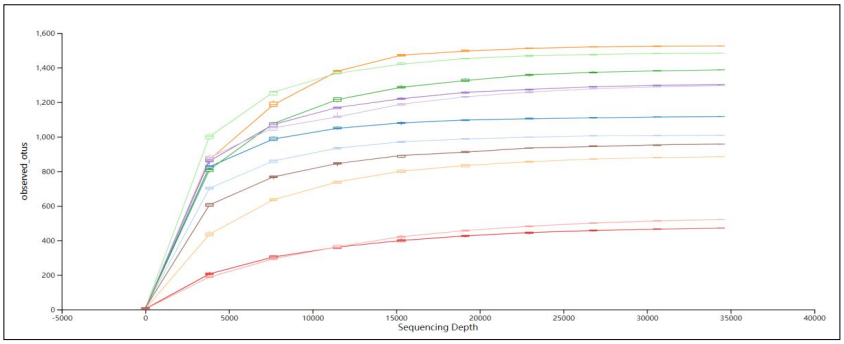

Fig 1: Sample dilution curves.

1.10%, respectively; likewise, the abundance of *Atoprostipes* was 1.46, 9.41, 6.38, 2.11, 3.78, 0.99 and 2.76%, respectively.

Furthermore, the Simpson index of the C sample was significantly lower than that of the F, B, P, T, N and W samples, indicating a significantly lower microbial diversity in milk samples from mastitic cows (Table 4). This suggested that mastitis changed the microbial diversity in cow milk.

There are many bacteria in milk, including some pathogenic bacteria such as *Staphylococcus aureus, Streptococcus and* pyogenic bacteria that may affect consumer health. Disinfection of cow nipples can prevent pathogenic bacteria from entering the raw milk. The milk-collecting cup comes in direct contact with the cow nipple. Therefore, the cleanliness degree of milk collecting cups can also directly affect the quality of raw milk. The dominant

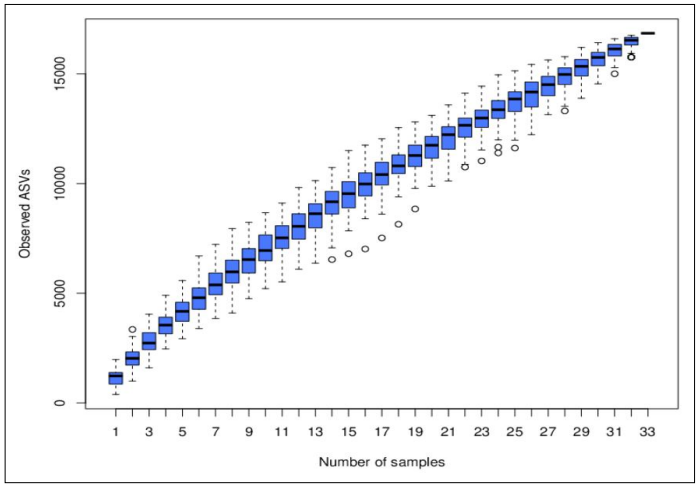

Fig 2: Species accumulation curve.

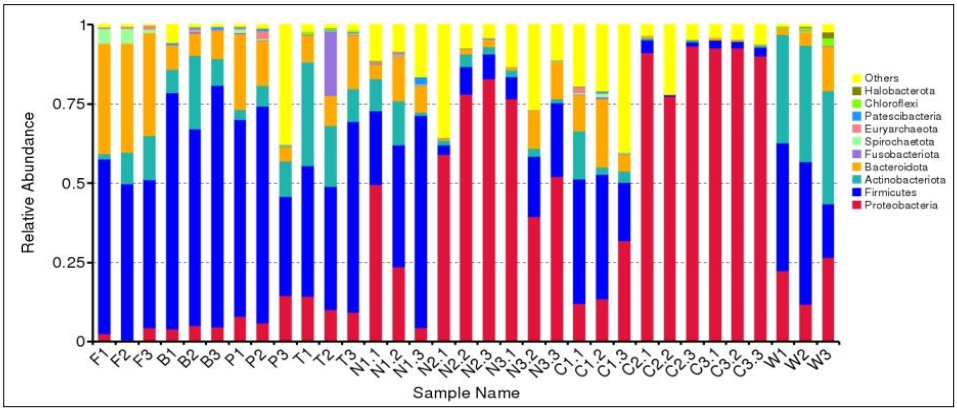

Fig 3: Relative abundance at the phylum level.

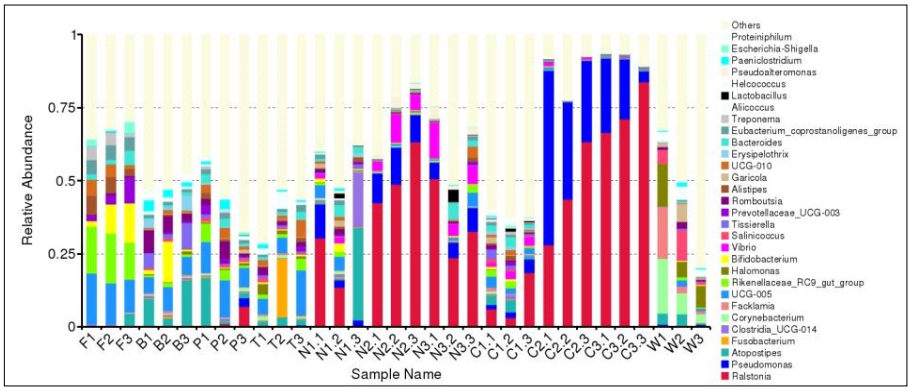

Fig 4: Relative abundance at the genus level.

annotated bacteria phyla in this study were Firmicutes, Proteobacteria, Bacteroides, *etc;* the dominant bacteria species were *Oscillospira-UCG-005, Atoprostipes, Pseudomonas*, *etc*. Our results are partially consistent with previous studies (Raats *et al.*, 2011; Rasolofo *et al.*, 2010;Delbeìs *et al.*, 2007; Qiang *et al.*, 2013). We found that the Simpson index of milk samples from mastitic cows was lower than in other samples. Also, the milk from mastitic cows had some pathogenic bacteria such as

Table 2: The relative abundance of microbiota at the phylum level in seven groups of samples.

Phylum	Groups						
	F	B	P	T	N	C	W
Proteobacteria	8.52%	12.95%	6.99%	20.79%	13.58%	13.79%	0.53%
Firmicutes	33.88%	7.82%	14.15%	11.56%	11.18%	12.30%	0.30%
Actinobacteria	0.00%	0.35%	0.27%	6.94%	2.52%	3.24%	0.06%
Bacteroidetes	3.50%	0.12%	0.60%	0.20%	0.31%	0.37%	0.00%
Fusobacteria	0.70%	0.35%	1.05%	0.15%	0.52%	0.57%	0.00%
Spirochaetes	0.06%	0.56%	0.28%	0.15%	0.33%	0.25%	0.01%
Euryarchaeota	0.00%	0.12%	0.14%	0.62%	0.29%	0.35%	0.00%
Patescibacteria	0.01%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%
Chloroflexi	0.47%	2.21%	13.13%	1.58%	5.64%	6.78%	9.87%
Halobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Others	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Table 3: The relative abundance of microbiota at the genus level in seven groups of samples.

Genus	Groups						
	F	B	P	T	N	C	W
Ralstonia	0.01%	0.10%	2.70%	0.10%	34.03%	42.72%	0.00%
Pseudomonas	0.09%	0.12%	1.10%	0.36%	7.42%	19.88%	0.71%
Atopostipes	1.46%	9.41%	6.38%	2.11%	3.78%	0.99%	2.76%
Fusobacterium	0.00%	0.32%	0.25%	6.93%	0.16%	0.07%	0.05%
Clostridia	0.63%	0.67%	0.50%	0.22%	2.35%	0.25%	0.02%
Corynebacterium	0.12%	0.46%	0.78%	1.03%	0.34%	0.16%	9.63%
Facklamia	0.02%	0.63%	0.18%	0.55%	0.12%	0.20%	7.02%
Oscillospira-UCG-005	14.25%	6.66%	9.91%	8.66%	1.82%	1.10%	1.10%
Rikenellaceae_RC9	15.28%	0.83%	3.66%	2.18%	0.73%	0.63%	0.42%
Halomonas	0.03%	0.65%	0.44%	1.98%	0.07%	0.06%	9.12%
Bifidobacterium	8.28%	5.40%	0.46%	0.48%	0.61%	0.24%	0.26%
Vibrio	0.00%	0.02%	0.03%	0.12%	4.91%	1.06%	0.16%
Salinicoccus	0.00%	0.55%	0.99%	0.71%	0.15%	0.30%	5.21%
Tissierella	0.06%	5.17%	0.72%	0.70%	0.27%	0.61%	0.31%
Prevotellaceae_UCG-003	4.58%	0.51%	1.67%	0.96%	0.34%	0.21%	0.07%
Romboutsia	0.46%	5.19%	2.98%	1.50%	0.45%	0.21%	1.09%
Alistipes	4.85%	0.32%	1.11%	0.79%	0.51%	0.34%	0.09%
Garicola	0.00%	0.27%	0.53%	0.22%	0.55%	0.30%	2.69%
UCG-010	3.73%	0.66%	2.36%	3.03%	0.78%	0.41%	0.11%
Erysipelothrix	0.01%	3.09%	0.38%	0.30%	0.18%	0.32%	0.08%
Bacteroides	2.74%	0.79%	2.29%	1.33%	1.74%	0.76%	0.29%
Eubacterium_	4.92%	1.06%	1.59%	1.43%	0.60%	0.33%	0.14%
Treponema	3.49%	0.12%	0.59%	0.18%	0.05%	0.20%	0.08%
Aliicoccus	0.00%	1.02%	0.77%	1.45%	0.12%	0.08%	3.57%
Lactobacillus	0.00%	0.00%	0.02%	0.00%	0.79%	0.16%	0.00%
Helcococcus	0.00%	0.10%	0.07%	1.49%	0.02%	0.00%	0.00%
Pseudoalteromonas	0.00%	0.00%	0.00%	0.00%	1.72%	0.35%	0.00%
Paeniclostridium	0.28%	2.58%	1.66%	0.85%	0.17%	0.09%	0.73%
Escherichia-Shigella	2.11%	0.02%	0.26%	0.03%	0.11%	0.12%	0.02%
Proteiniphilum	0.10%	2.13%	0.52%	0.17%	0.06%	0.56%	0.25%
Others	32.48%	51.13%	55.09%	60.13%	35.06%	27.28%	54.02%

Table 4: Diversity analysis of microbiota in 7 groups of samples.

Samples	F	B	P	T	N	C	W
Simpson index	0.9860±0.00	0.9840±0.00	0.9887±0.00	0.9783±0.0	0.8386±0.0	0.6721±0.1	0.9727±0.0
	493[a]	300[a]	176[a]	1299[a]	6740[ab]	6301[b]	1637[a]

Note: same small letters indicate no significant differences (P>0.05); different small letters indicate significant differences (P<0.05).

bacilli and cocci. Bacilli are human opportunistic pathogens. In case of lower resistance, these pathogens can easily cause body infection. Cocci, especially *staphylococcus aureus*, are the main pathogenic bacteria in lactating cows with mastitis, which are vulnerable to environmental changes. A study found that teats, bedding materials and milking equipment can be possible pollution sources for golden grapes (Lan, 2017). Oliveria *et al*. (2011) found that the washing degree of lactating cow teats before milking directly affected the *Staphylococcus aureus* content in fresh milk. Similarly, the bacterial content in the milk collecting cup can directly affect the quality of fresh milk. Also, disinfection/sterilization of the surface of the milk collecting turntable is very critical for the quality control of fresh milk.

The best way to prevent cow mastitis is to control or eliminate the source of harmful microorganisms. However, it is difficult to fully remove pathogenic microorganisms due to open areas in cow farms. Therefore, timely and accurate microbial analysis in dairy farms can provide early warning to establish safety measures.

CONCLUSION

This study showed that Proteobacteria and Firmicutes are the dominant bacteria in the milk collection system of dairy farms; *Oscillospira-UCG-005* and *Atoprostipes* were the dominant bacterial species. The bacterial diversity and richness significantly changed with farm sampling sites. Alpha diversity (Shannon index) of milk samples from mastitic cows was lower than in other samples. Overall, environmental disinfection of the farm and sterilization of milk collection equipment are critical for the prevention and treatment of cow mastitis.

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Conflict of interest: None.

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