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Microbial diversity analysis of subclinical mastitis in dairy cattle in Northeast China

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The microbial diversity of a pooled milk sample (H) from healthy dairy cows in the Daqing region of Heilongjing Province in northeastern China was compared to that of four pooled milk samples (M1-M4) from dairy cows from the same area previously diagnosed with subclinical mastitis, based on the California mastitis test, using pyrosequencing of the metagenomic *16S* ribosomal RNA genes. A total of 22 759 valid sequences were obtained from the five pooled milk samples, and the average length of a valid sequence was 484 bp. The composition of the bacterial communities of the M1, M2, M3, and M4 samples was significantly different from that of the H sample. The bacterial diversity of the M1 sample was highly similar to that of M2 sample; the bacterial diversity of the M3 sample was highly similar to that of M4 sample. The *Streptococcus* and *Staphylococcus* spp. were highly prevalent in the M1, M2, and M4 samples, and *Pseudomonas* spp. were highly prevalent in the M1, M2, and M3 samples. The levels of *Acinetobacter*, *Lactococcus*, and *Flavobacteriaceae* spp. in the M1, M2, M3, and M4 samples were lower than those of the H sample, which represents potential risk factors for subclinical mastitis. Our findings provide valuable information relevant to the prevention and treatment of subclinical mastitis in dairy cows in northeastern China.

Key words: subclinical mastitis, microbial diversity, pyrosequencing.

INTRODUCTION

Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle in cows-producing countries. Dairy cow mastitis causes economic losses due to reduced milk production, discarded milk, premature culling, and increased antibiotic usage (Halasa et al., 2007). The incidence rate of subclinical mastitis can reach 36.4%~85.7% on some diary farms in China (Ding et al., 2011). Compared with clinical mastitis, subclinical mastitis causes greater economic losses because of delays in treatment caused

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by the apparent lack of symptoms (Annapoorani et al., 2007).

Environmental microbial infections are considered to be the primary risk factors for mastitis in dairy cattle (Smith et al., 2005; Ericsson et al., 2009; Braem et al., 2012; Hogan and Smith, 2012). In previous decades, conventional bacterial culture, polymerase chain reaction (PCR), and PCR-denaturing gradient gel electrophoresis were widely used to identify and analyze the pathogenic bacteria associated with mastitis in dairy cattle (Kuang et al., 2009;Koskinen et al., 2010; Braem et al., 2011; Gurjar et al., 2012). In a recent study, Bhatt and others (2012) analyzed the microbiome signatures of milk samples from cows with subclinical mastitis using a shotgun pyrosequencing method, and Oikonomou et al. (2012) analyzed the microbial diversity of bovine mastitis-associated bacteria using pyrosequencing of the metagenomic 16s ribosomal RNA genes (16SrDNA). Metagenomic sequencing strategies obtain substantial information regarding the microbial composition of milk samples from dairy cows with mastitis.

The incidence of subclinical mastitis in dairy cows is closely related with environmental conditions. In different regions, the pathogenic bacteria associated with mastitis in dairy cattle may exhibit significant differences. In our current study, we used pyrosequencing of metagenomic 16S rDNA to analyze the microbial diversity of milk samples from dairy cows with subclinical mastitis in the Daqing area of Heilongjiang Province in northeastern China. Our findings provide valuable information for the prevention and treatment of subclinical mastitis in dairy cattle.

MATERIALS AND METHODS

Milk sampling

Twenty (20) milk samples (approximately 5 mL each) were collected aseptically in sterile centrifuge tube from Holstein cows with subclinical mastitis that were diagnosed using the California mastitis test (CMT) in 2013. The udder was washed thoroughly with potassium permanganate solution (1: 1000), and the teats were wiped with 70% ethyl alcohol prior to sampling. Based on their CMT rating, the CMT±, CMT+, CMT++, and CMT+++ cases were assigned to the M1, M2, M3, and M4 group, respectively, with five cows in each group. Milk samples from each cow were combined to form a pooled sample for the respective group. Milk samples were also collected from five healthy, unaffected cows from the affected dairy herd, and combined to form one pooled sample (H), which served as a control for the comparisons of microbial diversity. The H, M1, M2, M3, and M4 pooled samples were stored at -80°C.

DNA extraction, PCR amplification, and pyrosequencing

Genomic DNA was extracted from 1 ml of the H, M1, M2, M3, and M4 milk samples using the EZNA bacterial DNA isolation kit (Omega Biotek, Guangzhou, China), according to the manufacturer's instructions. The extracted bacterial genomic DNA was analyzed by electrophoresis on 1% agarose gel. The V1-V3 region of the 16S rDNA in each sample, which contains a unique 10-bp barcode sequence used for multiplexed pyrosequencing, was amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') oligonucleotide primers (Oikonomou et al., 2012). The PCR was performed in triplicate using 5 µl of 5× buffer, 0.5 µl of extracted genomic DNA, 0.4 µM each primer, 2.5 U TransStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China), 0.25 mM deoxynucleotide triphophates, and deionized water in a total volume of 25 µl. The protocol was optimized to reduce the number of cycles to ensure acceptable levels of accuracy and reliability in the subsequent data analysis. Thermal cycling was performed as follows: 95°C for 5 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. The PCR triplicates were pooled, and 2 µl of the pooled PCR products was analyzed on a 2% agarose gel. The PCR products were purified using a DNA gel extraction kit (Axygen, Hangzhou, China), and quantified using a QuantiFluor-ST Fluorometer (Promega, Madison, WI, USA). Pyrosequencing was performed from the A-end by a commercial service provider (Majorbio Bio-Pharm Technology, Shanghai, China) using a Roche 454A sequencing primer kit on a Roche GS-FLX Titanium platform genome sequencer (Roche Diagnostics, Indianapolis, IN, USA).

Bioinformatics analysis

The trimmed sequences were clustered to operational taxonomic units (OTUs) at phylotype similarity level of 97% by using the furthest neighbor method in the Mothur software package (www.mothur.org). Rarefaction analysis was performed using Mothur, and the rarefaction curve was generated using the R software (R Foundation for Statistical Computing, Vienna, Austria). The Shannon diversities and the Chao1 richness estimations were calculated using Mothur. The microbial-community barplot was generated using the R software, based on the data in the tax-level files. A heatmap figure and Venn diagrams were constructed using the R software. The principal component analysis (PCA) was performed to demonstrate clustering using the VEGAN computational tool in the R software. The similarity data were analyzed using the Jest method (Jaccard coefficient using richness estimators) and the Mothur software, and a similarity tree was generated using the R software.

RESULTS

Pyrosequencing of bacteria in milk from cows with subclinical mastitis

The 16S rDNA in the H, M1, M2, M3 and M4 samples were analyzed by metagenomic pyrosequencing of the V1-V3 region. A total of 22 759 valid sequences were obtained from the five pooled PCR samples, and the average length of a valid sequence was 484 bp (Table 1). After trimming and quality control, 20 470 sequences were obtained, and the average trimmed sequence length was 498 bp, accounting for 89.95% of the valid sequences. The number of the trimmed sequences in the H, M1, M2, M3, and M4 samples ranged from 3747 to 4464. A total of 1195 OTUs were obtained, with 97% sequence identity (0.03 cutoff distance) based on the Chao 1 and Shannon indices in Table 2. The OTUs ranged

Table 1. Valid sequences and trimmed sequences of bacterial 16S rRNA genes identified from milk samples with subclinical mastitis by pyrosequencing.

Sample	Valid sequences		Trimmed sequences		Percent (trimmed/valid)
	Sequence no.	Average length (bp)	Sequence no.	Average length (bp)	
H	5003	466	4339	496	86.72
M1	4393	491	3995	501	90.94
M2	4257	476	3747	498	88.02
M3	4820	492	4464	498	92.59
M4	4286	493	3925	498	91.58
Total	22759	484	20470	498	89.95

Table 2. Chao1 and Shannon indices for different cutoff distances (0.01, 0.03 and 0.05) and for different samples.

Sample name	Distance cutoff	Number of samples/sequences analyzed	Clusters (OTUs)	Chao1	Shanon index
	0.01		446	1075	2.71
H	0.03	1/4228	236	404	2.27
	0.05		173	257	1.97
	0.01		510	1168	4.00
M1	0.03	1/3963	301	583	3.38
	0.05		233	383	3.2
	0.01		452	1246	3.52
M2	0.03	1/3689	260	550	2.93
	0.05		203	350	2.75
	0.01		399	1168	2.37
M3	0.03	1/4449	201	407	2.02
	0.05		145	291	1.83
	0.01		382	1248	2.98
M4	0.03	1/3901	197	448	2.58
	0.05		141	254	2.39

from 197 to 301. The rarefaction curves showed a trend toward a saturation plateau, demonstrating the validity of the data with regard to a greater number of sequences producing fewer OTUs (Figure 1).

Classification and analysis of bacteria in milk from cows with subclinical mastitis

The bacterial community barplot of the H, M1, M2, M3, and M4 samples at the genera level are shown in Figure 2. The bacterial genera in the H, M1, M2, M3, and M4 samples included *Acinetobacter*, *Lactococcus*, *Pseudomonas*, *Sphingobacterium*, *Streptococcus*, *Staphylococcus*, *Empedobacter*, *Chryseobacterium*, *Flavobacterium*, and *Brochothrix*. The low-abundance bacterial genera in each sample were grouped as “others.” The relative abundance of bacterial genera among the five samples was significantly different, especially with regard to the levels of *Streptococcus* and *Staphylococcus* spp., which are commonly reported pathogens associated with mastitis in dairy cattle.

The hierarchical heatmap was generated based on the identified bacterial community in the samples at the genera level (Figure 3). The heatmap of the five samples indicated the existence of two different groups, one of

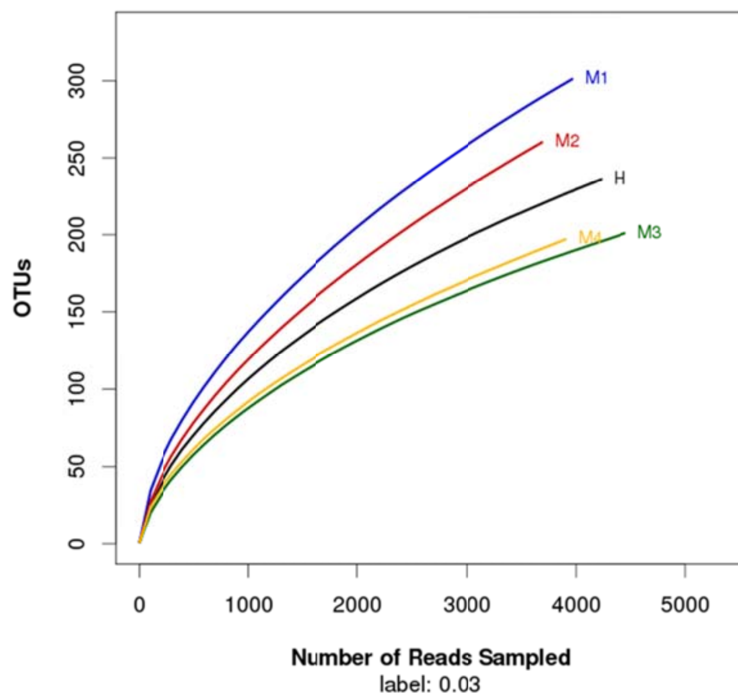

Figure 1. Rarefaction analysis of the H, M1, M2, M3, and M4 milk samples. Rarefaction curves of the OTUs clustered at a phylotype similarity level of 97%.

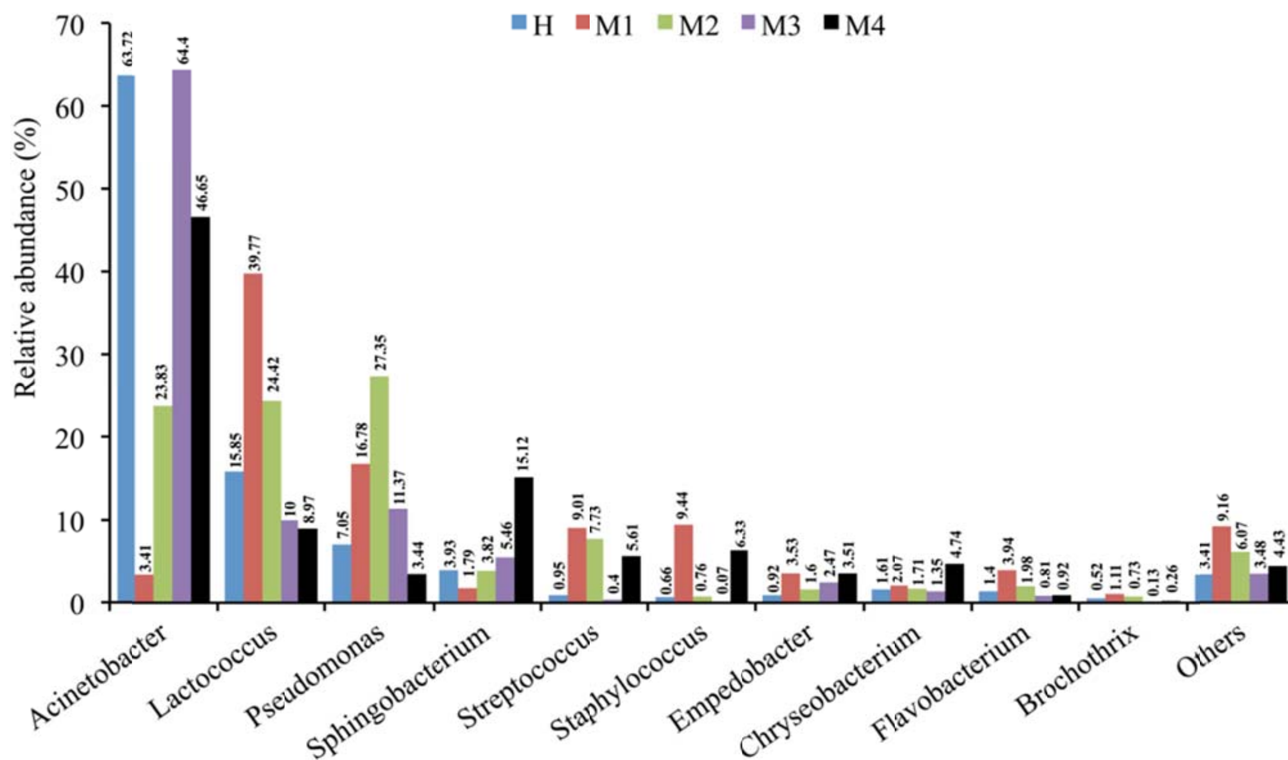

Figure 2. Bacterial community barplot of the H, M1, M2, M3, and M4 milk samples at the genera level. The relative abundance (%) was calculated based on the number of sequences of each bacterial genera in each sample.

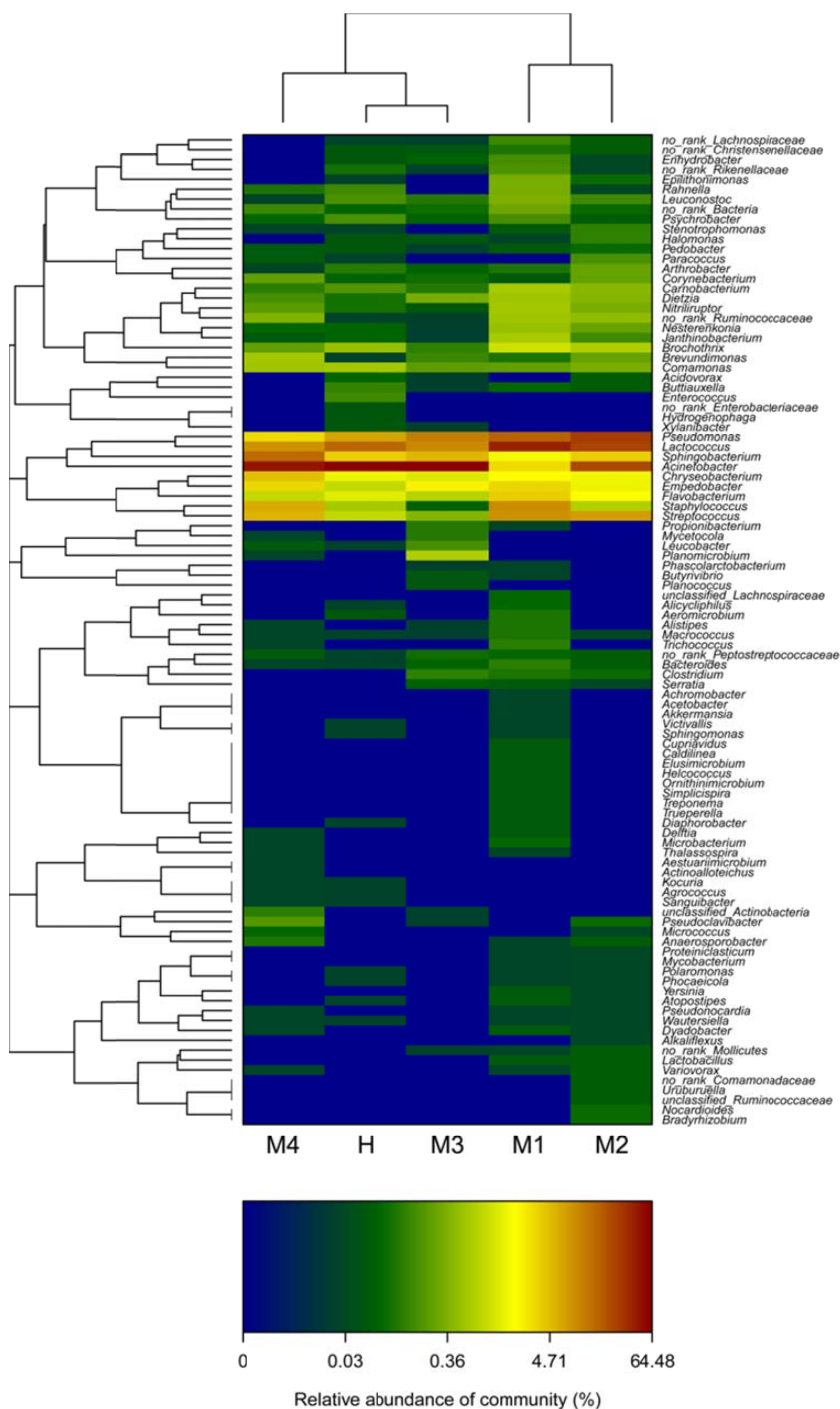

Figure 3. Heatmap figure of the bacterial-community composition of the H, M1, M2, M3, and M4 milk samples.

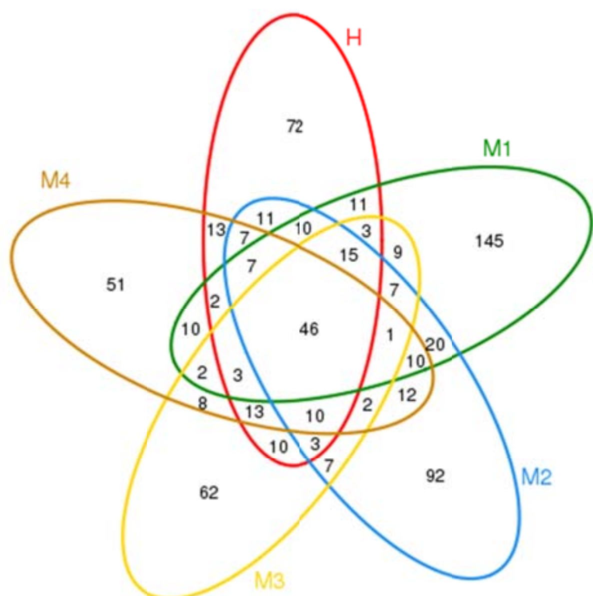

Figure 4. Venn diagram of the OTUs of the H, M1, M2, M3, and M4 milk samples at a distance level of 3%.

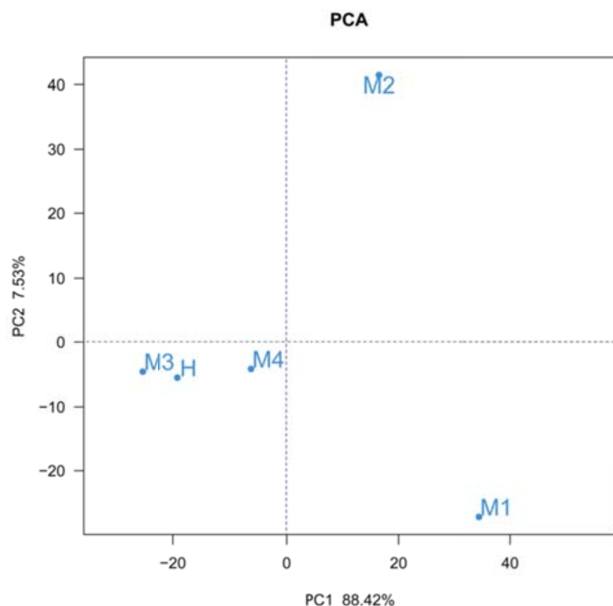

Figure 5. Scatter plot of the principal components analysis of the H, M1, M2, M3, and M4 milk samples. The PC1 and PC2 exhibited 88.42 and 7.53% of the variance, respectively.

which included the H, M3, and M4 samples, and the other included the M1 and M2 samples. The bacterial community of the H sample from the healthy dairy cows did not occupy a separate branch in the heatmap, and

Figure 6. Similarity tree of the H, M1, M2, M3, and M4 milk samples.

showed a high level of similarity with the M3 sample. The M1 sample had a high level of similarity with the M2 sample; the M3 sample had a high level of similarity with the M4 sample.

The shared communities among the five samples are depicted in the Venn diagrams in Figure 4. The shared OTUs of the M1, M2, M3, and M4 subclinical mastitis samples accounted for 4.9% of the 959 OTUs obtained for the four samples, of which the M1 and M2 samples shared the greatest number, at 116. The H, M1, M2, M3, and M4 samples shared 46 OTUs which accounted for 3.85% of the 1195 OTUs obtained for the five samples, and the shared OTUs of the H and M2 samples is 109.

The bacterial communities of the H, M3, and M4 samples were grouped in the bottom left of the PCA graph along the PC1, whereas the bacterial communities of the M1 and M2 samples were scattered throughout different areas of the PCA plot (Figure 5). Compared with the M1 and M2 samples, the bacterial communities of the H, M3, and M4 samples were more similar, which was consistent with the results of the heatmap analysis. In addition, the structure of similarity tree of the H, M1, M2, M3, and M4 samples was consistent with the PCA plot and heatmap (Figure 6).

DISCUSSION

Among the genera comprising the bacterial communities in the H, M1, M2, M3, and M4 samples, *Acinetobacter*, *Lactococcus*, *Empedobacter*, *Pseudomonas*, and *Chryseodobacterium* spp. have been reported as bacterial isolates from raw cow's milk (Hagi et al., 2013), and pathogenic *Streptococcus*, *Staphylococcus*, and *Flavobacterium* spp. have been reported in dairy cows (Rampon and Barbesier, 1960; Sela et al., 2007; Rato et

al., 2013; Wang et al., 2013). In addition, *Lactococcus* is an increasingly reported cause of bovine mastitis (Plumed-Ferrer et al., 2013). However, *Sphingobacterium* and *Brochothrix* spp., which were also identified in the five samples, have not been previously reported in dairy cows with mastitis.

Compared with the milk in the H sample from healthy cows, the proportions of *Lactococcus* (39.77%), *Pseudomonas* (16.78%), *Streptococcus* (9.01%), *Staphylococcus* (9.44%), *Empedobacter* (3.53%), and *Flavobacterium* (3.94%) were higher in the M1 sample, and the proportions of *Lactococcus* (24.42%), *Pseudomonas* (27.35%), and *Streptococcus* (7.73%) were higher in the M2 sample. In addition, the proportions of *Acinetobacter* (64.4%), *Pseudomonas* (11.37%), *Empedobacter* (2.47%), and *Sphingobacterium* (5.46%) in the M3 sample were higher than those in the H sample, and the proportions of *Sphingobacterium* (15.12%), *Streptococcus* (5.61%), *Staphylococcus* (6.33%), *Empedobacter* (3.51%), and *Chryseobacterium* (4.74%) in the M4 sample were higher than those in the H sample.

Streptococcus and/or *Staphylococcus* spp. were abundant in the M1, M2, and M4 samples, which is consistent with previous reports of *Staphylococcus* or *Streptococcus* associated with mastitis in dairy cattle (Saei et al., 2009; Kozytska et al., 2010; Piccinini et al., 2010; Demon et al., 2012). *Pseudomonas* spp., which was highly abundant in the M1, M2, and M3 samples, were reported to be associated with an outbreak of mastitis. In addition, the relative abundance of *Acinetobacter* was significantly lower in the M1, M2, and M4 samples, compared with that of the H sample. The relative abundance of *Lactococcus* in the M3 and M4 samples was lower than that of the H sample. Thus, the differences in the abundance of *Acinetobacter* and *Lactococcus* spp. may be related to the incidence of subclinical mastitis.

The *Flavobacterium*, *Empedobacter*, and *Chryseobacterium* genera belong to the family *Flavobacteriaceae*, which primarily include environmental bacteria. The presence of *Flavobacterium*, *Empedobacter*, and *Chryseobacterium* in the milk of cows with subclinical mastitis might provide novel information for the pathogenic bacteria associated with mastitis in dairy cattle. The Gram-positive firmicutes are generally considered as contagious mastitis pathogens, and the Gram-positive bacteria *Streptococcus* and *Staphylococcus* are the most common cause of bovine mastitis in China (Yang et al., 2013; Alkasir et al., 2013). In our current study, *Streptococcus* and *Staphylococcus* had the higher relative abundance in the milk of cows with subclinical mastitis. However, these genera comprised only 0.07% to 9.44% of the total bacteria in each sample. The relative abundance of *Streptococcus* and *Staphylococcus* in milk samples may provide novel understanding between these bacteria and both clinical and subclinical mastitis in dairy cattle.

Oikonomou et al. (2012) reported that high numbers of anaerobic bacterial sequences were obtained from all of the mastitis cases in their study using pyrosequencing of metagenomic 16s rDNA, suggesting a possible role of anaerobic pathogens, such as *Fusobacterium*, in bovine mastitis. In our current study, *Fusobacterium* spp. was not found in any of the milk samples. Such differences in the prevalence of anaerobic pathogens in mastitis cases might be associated with geographical differences. *Pseudomonas* spp. were highly abundant in three of the milk samples from cows with subclinical mastitis, suggesting that *Pseudomonas* spp. are a common cause of subclinical mastitis, which is consistent with a previous study of the microbiome signatures of milk samples from cows with subclinical mastitis (Bhatt et al., 2012).

The California mastitis test (CMT) is the most commonly used indirect method of evaluating somatic cell count. The ±, +, ++, and +++ scores of the CMT provide a qualitative assessment of subclinical mastitis in dairy cattle. In our current study, the M1, M2, M3, and M4 milk samples, which represented the ±, +, ++, and +++ CMT scores, respectively, exhibited different bacterial-community compositions. The M3 and M4 samples had similar bacterial communities, and the bacterial communities of the M1 and M2 samples were also similar. The bacterial-community composition of the H, M3, and M4 samples was clustered together in the PCA and the similarity tree. This finding suggests that subclinical mastitis may be caused by a variety of different pathogenic bacteria. Thus, other factors, such as viruses, may contribute to the development of subclinical mastitis.

In conclusion, our pyrosequencing analysis of 16SrDNA sequences revealed a high level of microbial diversity in the milk from cows with subclinical mastitis in northeastern China. Although the interpretation of our findings might be limited by the small number of milk samples tested and the small geographic area from which the milk was sampled, our results provide important basic information regarding microbial diversity that increase our understanding of the etiology of subclinical mastitis in dairy cattle.

Conflict of interests

The authors did not declare any conflict of interest.

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