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Analysis of Gut Fungal Community of Cows with Clinical Mastitis

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Abstract

Clinical Mastitis (CM) was one of the most common causes leading health disease in cows. In this article, we gave a new insight to gut fungal community of cows with CM. We chose two cows suffering from CM and four healthy cows from a local cow farm. We classified four healthy cows (H1, H2, H3, H4) into the control group and two cows (CM1, CM2) with CM into the case group. High-throughput sequencing was used to detect the difference of fungal community between the case group and the control group. The difference of gut fungi community was detected both at phylum and genus level. 4 phyla and 98 genera have been detected in the control group and the case group. At the phylum level, we found that the relative abundance of Basidiomycota in the case group was lower than that in the control group. At the genus level, the relative abundance of *Saccharomycetales*-unclassified and *Fungi*-unclassified were both higher whereas the relative abundance of *Pseudallescheria*, *Trichosporon*, *Microascaceae*-unclassified, *Candida* and *Scedosprium* in the case group was lower compared with the healthy group. Totally, the diversity and abundance of gut fungal community in the case group were lower than the control group. In conclusion, there are some differences of gut fungal community between the control group and the case group and the insights from this study could be used to develop a microbiota-based diagnosis for CM.

Keywords

Clinical Mastitis, Gut Fungal Community, Chinese Holstein Cows

1. Introduction

Mastitis, the inflammation of mammary gland, is one of the most prevalent dis-

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eases affecting dairy cows in worldwide [1]. Mastitis can be classified into three major types: Clinical Mastitis (CM), Sub-Clinical Mastitis (SCM) and Chronic Mastitis [2] [3] [4]. Among them, there is one common method for diagnosing CM by abnormalities including flakes, pus and the altering in color in the milk [5]. Because fungi can cause infections in animals [6], new methods of diagnosing CM have also been found from the perspective of observing the fungal community.

Fungi may sporadically infect cows through the injuries of the mammary gland caused by milking machine [7]. Extensive and indiscriminate use of antibiotics for treatment of mastitis can also make it easier for cows to encounter fungi invasion [8]. According to previous literature data, cows which are infected by fungi account for 2% - 13% of all the cows with mastitis [9] [10]. The incidence of mastitis due to fungi has also been reported by various workers [11] [12].

Many relevant studies have been focused on the fungal community in the cows with CM. Spanamberg [6] has found that *Candida* species are the most frequent organisms among the fungal community isolated from infected glands in cows with CM. Among them, the occurrence of *Candida tropicalis* is most frequent in clinical cases of mastitis [6] [11]. Besides, some researchers study the cows with CM and come to the conclusion that *Aspergillus spp.* was one of the causative fungi pathogens [13] [14]. *Geotrichum candidum*, *Trichosporon cutaneum*, *Saccharomyces cerevisiae* and *Rhodotorula rubra* have also been detected in the cows with CM [15].

In the literature published previously, the fungal community of the raw milk [15] and the rumen fluid of cows with CM have already been studied [16]. Despite researchers are aware of the severity of the Clinical Mastitis caused by fungi, few studies have been conducted in the cows to better understand how CM can affect the gut fungal community. It is well known that the gastrointestinal community plays an important role in maintaining host health [17]. And the dysbiosis of the gut fungal community can lead to high incidence of CM in cows and cause huge economic loss in milk yield [18] [19] [20]. Therefore, the fungal community of gut in cows with CM should be described in detail for further diagnosis. The present study is undertaken to detect the difference of gut fungal community between healthy cows and individuals with CM.

2. Material and Methods

2.1. Sample Collection

We picked out six Chinese Holstein cows from a specific farming cooperative located in Xuzhou. Among them, two cows with swelling in the mammary gland were diagnosed as the CM. We classified those two cows into the case group. Four healthy cows were classified into the control group.

2.2. DNA Extraction and Sequencing

The whole six cows were fed with the same green ecological within a week. On

the eighth day, fresh fecal sample were immediately collected by the animal raiser when each animal was upon defecation in the morning. Each stool sample was collected with the help of sterile cups and sterile cotton swabs, which was sent to the laboratory with dry ice, and processed immediately after arrival. All samples were stored at −80˚C before DNA extraction. Total genomic DNA was extracted by using the Stool DNA Extraction Kit (Omega, USA) following manufacture instruction. The extracts from the samples were pooled, extracted purity of DNA was verified by electrophoresis on ethidium bromide staining 1% agarose gels and concentration was analyzed spectrophotometrically using the M200pro (TECAN, Switzerland). Deep sequencing was performed on Illumina sequencing with MiSeq using paired-end technology provided by Shanghai Majorbio Co. Ltd, China. We amplified both the nrDNA ITS1 region of fungi using universal primer ITS1-2 (94˚C for 3 min, followed by 28 cycles of 94˚C for 30 s; 53˚C for 40 s and 72˚C for 1 min; after which a final elongation step at 72˚C for 5 min was performed). The DNA samples were then quantified according to the manufacturer's instructions and quality controlled on a QuantiFluor™-ST blue fluorescence quantitative system (Promega, USA). Following quantitation, purified amplicons from each reaction mixture were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

2.3. Bioinformatics Analysis

All sequencing reads were filtered according to barcode and primer sequences using Usearch (vsesion 7.1 http://drive5.com/uparse/) software. The resulting sequences were further screened and filtered for quality. Sequences that were shorter than 50 bp in length and single sequences were removed. The remaining sequences were assigned into operational taxonomic units (OTUs) by 97% similarity. The OTUs were classified by Ribosomal Database Project (RDP) classifier. The following was comparing data according to ITS fungi database—Unite (Release 6.0 http://unite.ut.ee/index.php). Relative abundance of the main phyla and genera were calculated. We also calculated the coverage percentage, abundance based coverage estimator (ACE), Chao index, and the Shannon and Simpson diversity indices using the MOTHUR program (https://www.mothur.org/).

3. Results

The Shannon Wiener curves generated by MOTHUR plotted the number of qualified sequencing reads. It reflected the gut fungal diversity of samples. The curves tend to approach a horizontal asymptote, which indicated the sequencing effort was saturated. We classified cows named CM1, CM2 into the case group. Besides, the control group included four cows named H1, H2, H3, and H4. As was depicted in **Figure 1**, the diversity of the gut fungal community in the case group was lower than that in the control group.

Detailed information of six cows from which fecal samples were collected was presented (**Table 1**). We could get the times of calving of six cows from the row

Table 1. Characteristics of six Holstein cows.

Identifier	Characteristics of six Holstein cows					
	H1	H2	H3	H4	CM1	CM2
Weight/kg	610	580	590	580	609	600
Body length/m	1.73	1.70	1.71	1.69	1.72	1.75
Age/year	5	5.5	4.8	5.2	5.6	5
Parity	3	3	3	2	2	2

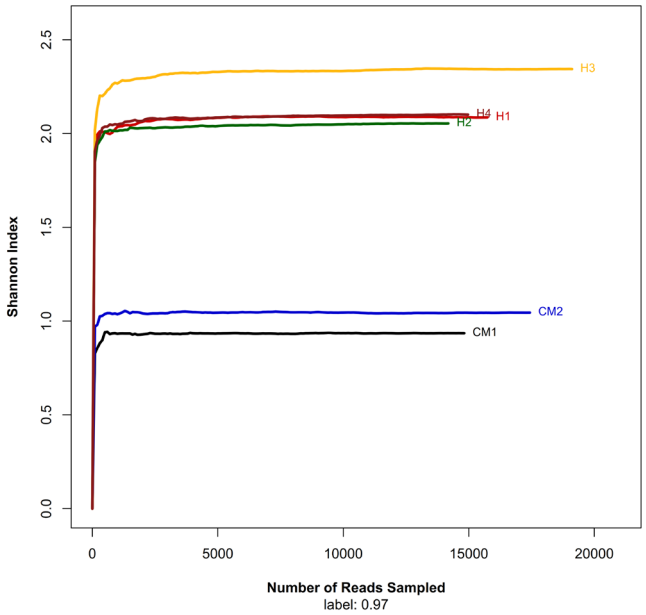


Figure 1. Shannon Wiener curves.

of parity clearly. The total number of OTUs, sequences, coverage and statistical estimates of species richness and diversity of gut fugal community were presented in **Table 2**. The Chao and Ace indices showed that the richness of gut fungal community in the case group was lower than the control group. What's more, Simpson and Shannon indices confirmed that there was lower level of gut fungal community diversity in the case group.

The rank-abundance distribution curve could be used to visualized species richness and species evenness. The curves of the case group were kept in a steep gradient, indicating low evenness as the high-ranking species have much higher abundance than the low-ranking species. Conversely, it was also clear that the OTU rank of CM1 and CM2 were lower, the curves of the control group was kept in a shallow gradient, indicating high evenness as the abundance of different species were similar (**Figure 2**).

All sequences were classified in phylum and genus using the program Mothur with the default setting. At the phylum level, the relative abundance of Ascomycota and Zygomycota in the case group was similar to the control group. Ascomycota was dominant both in the case group and the control group. However,

Table 2. Community diversity estimator.

Sample ID	Community diversity estimator						
	Reads	OTU	Ace	Chao	Coverage	Shannon	Simpson
H1	15,744	133	146	147	0.998666	2.09	0.2777
H2	14,182	116	133	129	0.998308	2.05	0.2143
H3	19,112	144	148	149	0.999372	2.34	0.1829
H4	14,969	129	142	137	0.998597	2.10	0.2251
CH1	14,817	42	78	58	0.999123	0.94	0.6433
CH2	17,434	49	60	55	0.999369	1.05	0.5962

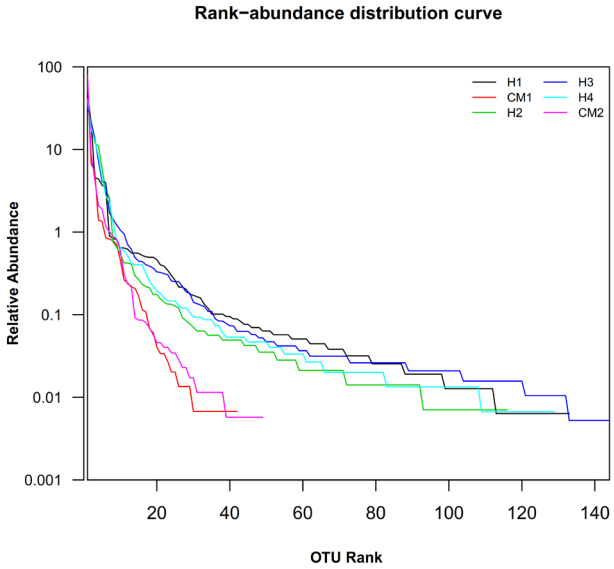
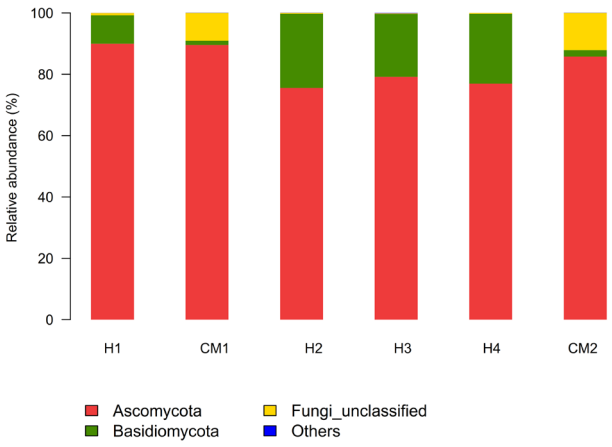


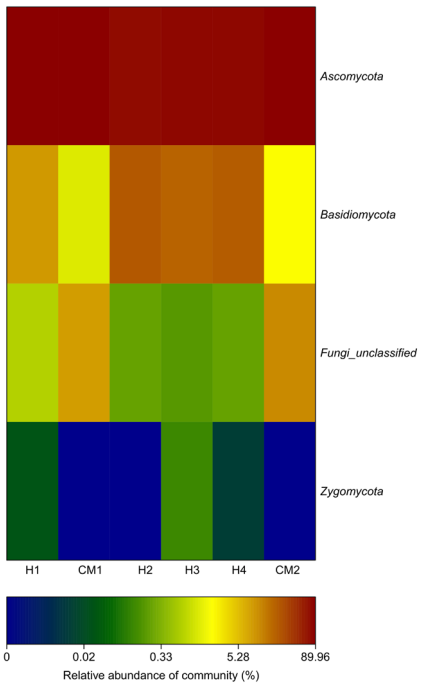
Figure 2. Rank-abundance distribution curve of six cows. X-axis reflected the rank of OUT according to the relative abundance, Y-axis reflected the relative abundance of OUT. Each curve represented an individual cow. CM = Clinical Mastitis, H = Healthy.

the relative abundance of Basidiomycota was lower and the relative abundance of unclassified fungi was higher in the case group compared with the control group (**Figure 3(a)**, **Figure 3(b)**).

Main fungi genus have been detected and heatmap was generated to graphically represent the relative abundance of gut fungal genera. *Pseudallescheria* was dominated followed by *Microascaceae*-unclassified in the control group. In the case group, the proportion of many unclassified species which belonged to *Saccharomycetales* were in highest in the case group. It may suggest that there may be a wide range of *Saccharomycetales* disorders in the gut fungal community of cows with CM. What's more, the relative abundance of *Pseudallescheria*, *Microascaceae*-unclassified, *Scedosporium* and *Trichosporon* were lower than the control group (**Figure 4(a)**, **Figure 4(b)**). From the figure presented above, the diversity of gut fungal community in the case group was lower than the control group at the genus level.



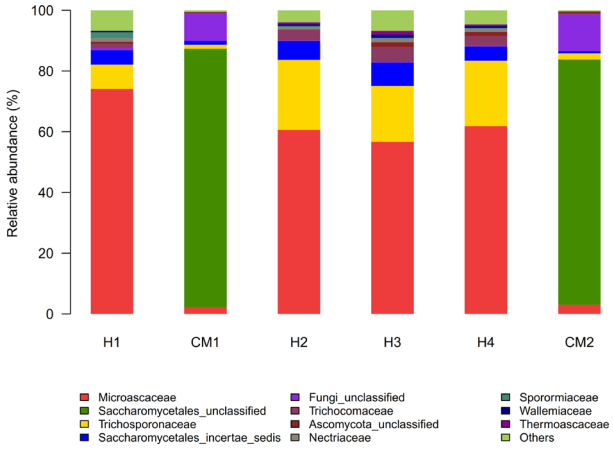
(a)



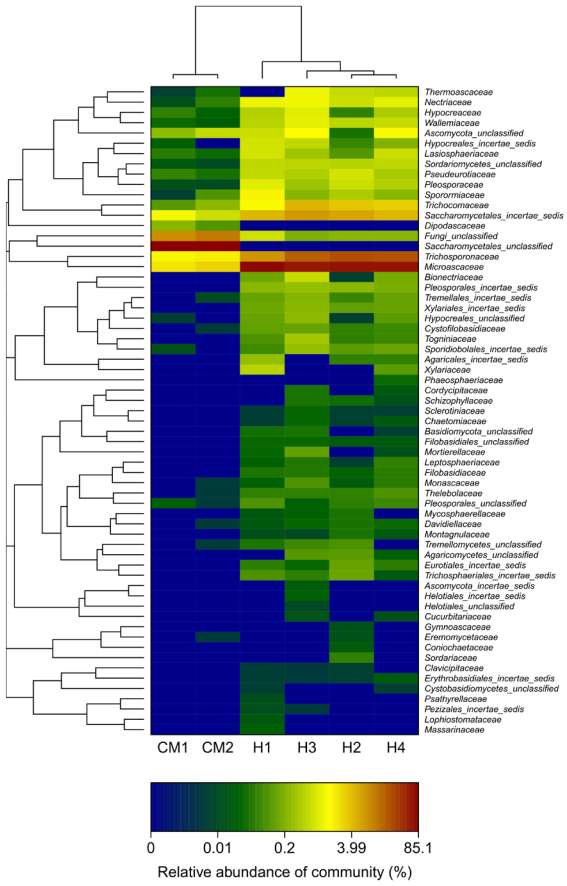
(b)

Figure 3. Relative abundance of the main fungi phyla in healthy and with CM cows presented by bar plot (a) and heatmap (b). The heatmap plot depicts the relative percentage of each fungal phylum (b). Each row represents phylum, and each column represents an individual cow (a), (b). CM = Clinical Mastitis, H = Healthy, others represent genera whose relative abundance below 1%.

Based on the principal component analysis, the fungal communities of healthy cows and cows with CM showed distinction (**Figure 5(a)**). The fungal communities of CM1, CM2 clustered closely and didn't cluster with H1, H2, H3, H4. As the results revealed, H1 wasn't close to the other three healthy cows H2, H3, H4. We attempted to make Hierarchical clustering analysis according to beta diversity distance matrix. And then use unweighted pair group method with arithmetic mean to build tree structure. The length of branch represented the



(a)



(b)

Figure 4. Relative abundance of the main fungi genera in healthy and with CM cows presented by bar plot (a) and heatmap (b). The heatmap plot depicts the relative percentage of each fungal genus (b); Each row represents phylum, and each column represents an individual cow (a), (b). CM = Clinical Mastitis, H = Healthy, others represent genera whose relative abundance below 1%.

distance between samples and vertical bar in the end represent clustering [21] (**Figure 5(b)**). In conclusion, Principal coordinates analysis and the multiple

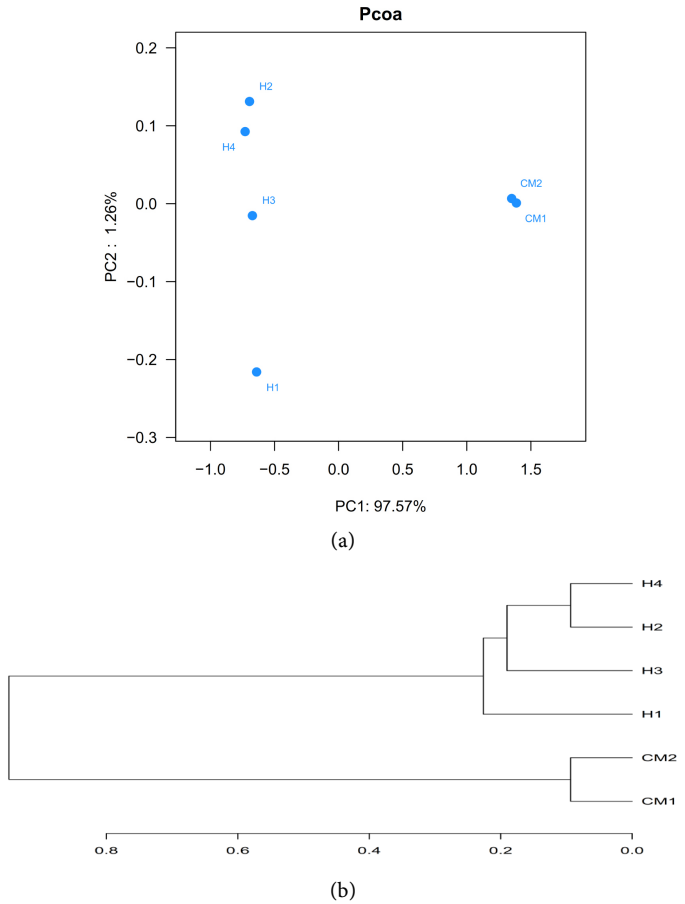


Figure 5. (a) Two-dimensional PCoA plot illustrates variation in fecal fungal community as affected by Clinical Mastitis (CM); H = healthy; (b) Multiple samples similarity tree.

samples similarity tree showed that the gut fungal community of the case group was clearly distinguishable from that of the control group at the level of the OUT (**Figure 5(a)**, **Figure 5(b)**).

4. Discussion

Product safety and animal welfare in dairy management system can be improved by means of early detection of diseases such as mastitis [22]. Since the heritability for CM was extremely low [23], hardly can we predict the occurrence of CM according to genetic heritability. In addition, somatic cell counts (SCC) becomes the most common measurement of milk quality and udder health [24]. SCC is generally both an indicator of CM. It has been indeed demonstrated that mastitis causes an increase in SCC of cattle [25]. Though it is easy to identify Clinical Mastitis, more precise detection tests need to be explored.

In our study, firstly we found the difference of intestinal fungal community between the case group and the control group without limitation of observing bovine mammary gland tissue. Compared with the control group, the gut fungal community showed less diversity and richness in the case group. The result can be explained by the different relative abundance both in phylum and genera.

In phylum, the relative abundance of Basidiomycota in the case group was lower than the control group. The relative abundance of Ascomycota and Zygomycota in the case group was similar to the control group, which showed that the relative abundance of Ascomycota and Zygomycota were stable.

In genus, in comparison to the control group, the relative abundance of *Saccharomycetales*-unclassified was higher. On the contrary, the relative abundance of *Pseudallescheria*, *Microascaceae*-unclassified, *Scedosporium* and *Trichosporon* were lower than the control group. In the present work, it is found that a large number of unclassified genera and phyla in the feces of cows belong to unclassified, since little work on gut fungal community has been done. Although the incidence of CM caused by *Candida spp.* was high [6], the relative abundance was lower in the case group than the control group. It could be concluded that another fungi was the main pathogeny instead of *Candida*. Among them, unclassified species which belonged to *Saccharomycetales* was dominant, showing that it may be the core genus in the case group.

Nowadays, papers about mastitis and its optimal technical management and treatment are available [26] [27]. The prevalence of mastitis in organized dairy farm is decreased by improving preventive measures and using antibiotics. However, it is still of a challenge as many of these fungi do not respond to the antibiotics rather they use some of the antibiotic like tetracyclineas their source of energy [28]. Hence, it is important to study out new treatment according to the specific fungal community in cows with CM mentioned above.

This study used high-throughput sequencing technology is more comprehensive and systematic to measure the fungi in the feces of Chinese Holstein cows. The results suggest that Clinical Mastitis can cause the shifts of gut fungal community, showing that fungi diversity and abundance in the feces of Chinese Holstein cows with CM are lower than healthy cows with CM under the same diet.

5. Conclusion

The diversity and richness of gut fungal community in the case group are lower than those in the control group. It is found that Clinical Mastitis gives rise to gut fungal community disorder, which allows us to detect CM according to the microbial difference in phyla and genera. As a consequence, fungal detection of CM can be helpful to recover benefits of dairy cooperation and reduce the mortality of cows caused by CM. However, the relative abundance of unclassified fungi is predominant in the case group both in phylum and in genus. Thus, further study on fungal mastitis pathogens and relevant methods for treatment needs to be continued.

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