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RESEARCH ARTICLE

Microbial Diversity in Milk from Holstein Dairy Cattle with Mastitis in Southern China using Illumina MiSeq-Based Analysis

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ABSTRACT

To evaluate the microbial population in milk from dairy cows with mastitis in Guangxi Province, China, 11 fresh milk samples were collected from cows with mastitis at a farm in the province with 1000 Holstein dairy cows. A CMT was performed on the milk samples, and they were classified by parity: A (1_{th}), B (2_{nd}), and C (3_{rd}). The microbial community was analyzed via deep DNA sequencing of the bacterial 16S rRNA genes using the Illumina MiSeq platform. The results revealed that there were many bacteria and fungi present in the milk samples. Ten bacterial phyla (Acidobacteria, Actinobacteria, Bacteria-unclassified, Bacteroidetes, Candidate-division-TM7, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes) were identified. Firmicutes was the predominant phylum, followed by Tenericutes. The fungi found in the samples belonged to 2 phyla (Ascomycota and Basidiomycota). At the genus level, the most abundant bacterial operational taxonomic units (OTUs) were *Enterococcus* and *Mycoplasma*. The most abundant fungal genus was *Malassezia*, followed by Agaricales-unclassified. The data indicated that the predominant phylum in the milk samples was associated with climate, antibiotic resistance, and parity. In this study, we provide a theoretical foundation for research on the prevention of mastitis as well as the selection of medicine for mastitis treatment.

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INTRODUCTION

Mastitis is a common infectious disease in dairy herds and is defined as inflammation of the mammary gland resulting from infection with pathogenic microorganisms (Memon *et al*., 2013). Mastitis leads to huge economic losses, which include reduced quantity and quality of milk, premature culling, treatment costs, and discarded or low grade milk. Moreover, mastitis is also of public health significance (Hoque *et al*., 2015). There are many kinds of microorganisms that cause mastitis. The common pathogens are *Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, E. coli*, and *Mycoplasma*, as well as various species of yeast and fungi (Hertl *et al*., 2014). Previous studies showed that management was closely associated with mastitis (Khan *et al*., 2013; Ali *et al*., 2014). In addition, antibiotic therapy leads to the emergence of antibiotic resistant strains, such as *Staphylococcus aureus, E. coli*, and *Mycoplasma* (Caswell and Archambault, 2007). Meanwhile, many new mastitis-causing, antibiotic resistant bacterial strains have appeared, such as methicillin-resistant *Staphylococcus aureus* (Silva *et al*., 2014). Individually or combined, these reasons can cause bovine mastitis. Currently, there are several common methods for the diagnosis of mastitis, including clinical examination, imaging techniques, and the California mastitis test (CMT) (Fragkou *et al*., 2014; Hoque *et al*., 2015). Furthermore, the mastitis-causing pathogens can always be identified by bacterial culturing or fluorescent in situ hybridization (FISH) (Braem *et al*., 2012; Gey *et al*., 2013). However, these methods are not able to comprehensively detect microorganisms. Thus,

choosing medicines and developing new drugs for treatment became difficult. Therefore, we used the MiSeq platform to determine the microbial diversity because a large amount of sequencing can be performed, which allows the identification of more strains, thus providing a basis for choosing medicine and developing new drugs to treat mastitis. Sequencing technology has been used to study microbial populations in many fields, including human medicine, activated sludge, and gastrointestinal tracts (Ye and Zhang, 2007), as it provides a rapid, effective and economical way to better understand the microbial community (Green and Bradley, 2013).

Guangxi Province is located in southern China and is considered to be a warm, wet place in the subtropical zone. The environment, with warm temperatures and high humidity, is suitable for the development of cow husbandry. However, studies have shown that the high humidity and warm temperatures are associated with a high prevalence of bovine mastitis, which has constrained the development of the dairy industry (Li *et al*., 2014).

The prevalence of mastitis is a problem for both veterinarians and researchers (Qayyum *et al*., 2016). In this study, milk samples were collected from Guangxi Province and tested by CMT. The microbial diversity of the samples was assessed through deep DNA sequencing of the bacterial 16S rRNA genes using the MiSeq platform to detect the predominant microbial community members.

MATERIALS AND METHODS

Study area, animals and management: This study was conducted from 2012 to 2015 in Guangxi Province (longitude 108.22°E, latitude 22.48°N). The average rainfall was 1180.3 mm, the average temperature was 20.47°C, and the average relative humidity was 79.07% from 1985 to 2014 (data from the Guangxi Zhuang Autonomous Region Meteorological Science Data Sharing Service Center). There were 1000 Holstein dairy cattle in total. They were kept under housing and management in good condition. At this farm, when suffering from clinical or subclinical mastitis, the cows were treated daily with antibiotics, including potassium penicillin, streptomycin sulfate, and gentamicin sulfate.

Sample collection and detection of mastitis: At this farm, a total of 1000 dairy cows were sampled, and the milk collected during 2015 was tested using the CMT (Qayyum *et al*., 2016). The incidence of mastitis is shown in the results section. Among these cows, eleven milk samples (10 ml) were collected from different Holstein cows with clinical mastitis at 2-3 weeks postpartum in 2015 (Table 1) and were tested with the California mastitis test. These eleven dairy cows had clinical symptoms of red nipples and udder fever. The milk samples (10 ml) appeared flocculent with larger clot particles and were collected by hand milking under sterile conditions into a tube for each animal; the samples were then sent to the laboratory under conditions of low temperature and were stored at -80°C until ready for analysis. These samples were classified by parity: A (1_{th}), B (2_{nd}), and C (3_{rd}).

DNA extraction, PCR amplification, and amplicon quantification, pooling, and sequencing: Microbial DNA was extracted from 5 ml of the milk samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Inc, USA. Catalog no. D5625-02) according to the manufacturer's instructions. Bacterial 16S rRNA from the V3-V4 hypervariable region was amplified using forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'); a 10-nucleotide barcode was included in the forward primer. Fungi universal primers 0817F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and 1196R (5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the SSU region of fungal 18S rDNA. The PCR amplification reaction mixture contained 0.2 µl of rTaq DNA polymerase (TaKaRa, CA, USA), 2.5 µl of 10×Buffer, 2 µl of 2.5 mM dNTPs (TaKaRa, Dalian, China), 0.8 µl of 5 µM barcoded primer, 0.2 µl of BSA and 10 ng of genomic DNA template for a total volume of 20 µl. PCR was performed with a thermal cycler (ABI Gene Amp® 9700, USA) under the following condition: 3 min at 95°C, followed by 27 (bacterial) or 34 (fungal) cycles of 30s at 95°C for 30s, 55°C for 30s, and 72°C for 45s, and finally 10 min at 72°C. The PCR products of same sample were mixed within a PCR tube and were then visualized on agarose gels (2% in TBE buffer) containing bromide and purified with a DNA gel extraction Kit (Axygen, USA). Two hundred nanograms of purified amplicon from each sample were then mixed.

Measurement of bacterial abundance and community structure: The DNA extracted from the milk samples was subsequently sequenced using the Illumina MiSeq platform by Majorbio Bio Tech Co. Ltd. (Shanghai, China). The complete datasets were submitted to the NCBI Short Read Archive database under accession No. PRJNA295906.

RESULTS

Incidence of mastitis in this farm: From 2012-2015, samples were collected from Holstein cows in Guangxi province, China, chosen from 1000 lactating dairy cows (approximately 450 milking cows), and the average yearly incidence of mastitis using the CMT and elimination rate were 11% (50) and 2.2% (10), respectively.

Characteristics of MI sequencing results: In this study, bacteria were detected in 11 milk samples, and fungi were detected in 5 milk samples. For bacteria and fungi, 460024 and 182296 raw reads were obtained, respectively. After removing reads that contained incorrect primer or barcode sequences as well as sequences with more than one ambiguous base from the 11 samples through MI sequencing analysis, there were 204803 high-quality bacterial reads and 80814 fungal reads. The length distribution of the bacterial sequences was (Fig. A1) concentrated at 401~500 bp (99.85%), and the fungal sequences were (Fig. B1) concentrated at 301~400 bp (96.81%).

Richness and diversity: A total of 86 bacterial OTUs (Table 1) and 8 fungal OTUs were obtained from the 11

samples. Samples in group A contained OTUs from 40 to 47, group B contained OTUs from 37 to 65, and group C contained OTUs from 49 to 58. The rarefaction curves based on the OTUs (Fig. 2) showed that all the milk samples tended to approach the saturation plateau. The Good's coverage of all the milk samples was estimated to be over 99% (Table 1), indicating that the 16S and 18S rRNA sequences identified in these samples represent the majority of microbes in the samples. Richness diversity (Table 1) was calculated by estimating the number of OTUs based on the ACE and Chao estimators. For bacteria, among the samples of group A, the ACE and Chao values were lower than the values in group C, but the ACE values of group B were higher than the values of group C (except B3), and the Chao values of group B were higher than group C (except B5).

Microbial community structure of the milk samples: All sequences were classified from phylum to genus according to the program Mothur, and 10 different bacterial phyla (Acidobacteria, Actinobacteria, Bacteria-unclassified, Bacteroidetes, Candidate-division-TM7, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes) (Fig. A3) and 2 fungal phyla (Ascomycota and Basidiomycota) were identified. For bacteria (Fig. A3), the dominant phyla in all the groups were Firmicutes and Tenericutes. Each of the three groups (A, B, and C) was numerically dominated by Firmicutes (34.87%, 78.80%, and 72.4%, respectively) and Tenericutes (64.58%, 19.8%, and 25.70%, respectively). The most important fungal genus (Fig. B3) was *Malassezia*, followed by Agaricales-unclassified and Saccharomycetales-unclassified. Heatmap analysis (Fig. B4) showed that the OTU distribution unambiguously. The ten most abundant OTUs distributed in the different samples were determined to better understand which bacteria and fungi are important. The most abundant bacterial OTUs were *Enterococcus*, *Mycoplasma* and Entomoplasmataceae-Incertae-sedis (Fig. A3), while the most abundant fungal OTU was *Malassezia* (Fig. B3). The clustering results (Fig. A3) indicated that group B (except B5) and group C were more similar to each other than to group A (except A3), but B5 and A3 were similar to each other.

The relationship of parity, health condition and predominant microbes: The results (Table 2) show the relationship of parity, health condition and predominant microbes. The out and repeated recurrence correlated more with parity for the dairy cows with bovine mastitis. The history of mastitis also increased. The third and the fifth parity (group B and C) were dominated by Firmicutes (except B5), but, for group B, the most abundant bacterial genus was *Enterococcus*. The first parity (group A) was dominated by Tenericutes (except A3), and B3 and A3 were also dominated by Tenericutes, but their most abundant bacterial genus was *Mycoplasma*.

DISCUSSION

It has been reported that sequencing technology has a greater capacity to explore microbial richness than culture-dependent, fluorescent in situ hybridization (FISH) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methods, which indicates that sequencing technology is an efficient way to investigate microbial communities (Fragkou *et al*., 2014). Our results obtained by high-throughput sequencing technology showed that there were 12 different phyla, 64 genera of bacteria and fungi, which were higher numbers than those detected in previous studies of mastitis (Braem *et al*., 2012; Gey *et al*., 2013).

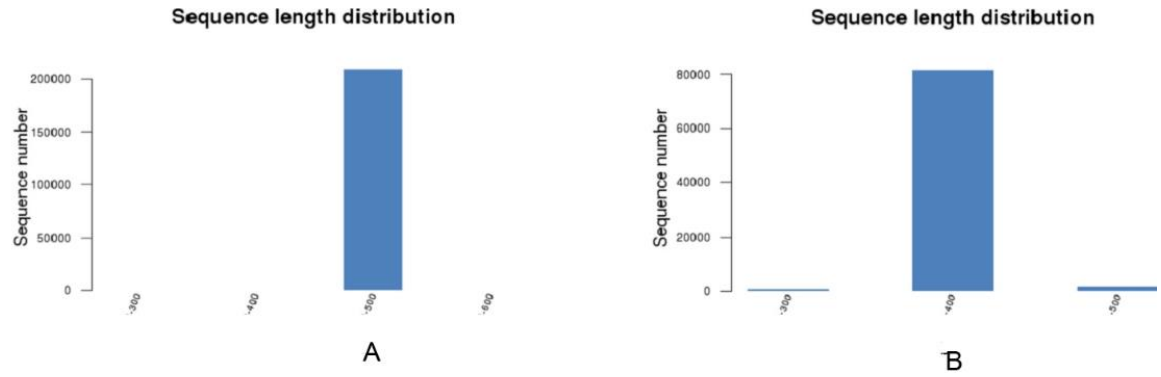

Fig. 1: Sequence length distribution. The sequence length of the bacterial reads was concentrated at 401-500 bp (A). The sequence length of the fungal reads was concentrated at 301-400 bp (B).

Table 1: Species richness estimates of the milk samples. A1~A3 (1th parity), B1~B5 (2nd parity), and C1~C3 (3rd parity)

Sample ID	Reads	0.97					
		OTU	ace	chao	coverage	shannon	simpson
A1	20311	45	50(46,64)	49(46,63)	0.999606	1.12(1.11,1.14)	0.4605(0.4548,0.4662)
A2	17745	47	52(48,64)	51(48,67)	0.999549	1.23(1.21,1.25)	0.4247(0.4191,0.4303)
A3	18822	40	55(45,86)	53(43,91)	0.999362	0.99(0.97,1.01)	0.5521(0.5445,0.5598)
B1	17763	65	72(67,88)	74(67,102)	0.999381	1.33(1.3,1.35)	0.5162(0.5073,0.5251)
B2	20134	57	60(58,71)	61(58,79)	0.999702	1.33(1.3,1.35)	0.5153(0.507,0.5237)
B3	17193	48	54(50,70)	52(49,68)	0.999535	1.41(1.39,1.43)	0.4738(0.4649,0.4827)
B4	19145	63	64(63,71)	65(63,75)	0.999791	1.35(1.33,1.38)	0.518(0.5094,0.5266)
B5	20446	37	76(56,116)	50(40,88)	0.999413	1.13(1.12,1.15)	0.3928(0.3894,0.3962)
C1	17291	58	67(61,86)	63(59,79)	0.999364	1.54(1.52,1.57)	0.3772(0.3701,0.3843)
C2	17503	49	54(50,67)	52(49,64)	0.999600	1.69(1.67,1.71)	0.2967(0.2912,0.3023)
C3	18450	54	60(56,73)	60(55,81)	0.999512	1.59(1.57,1.61)	0.3468(0.3407,0.3528)

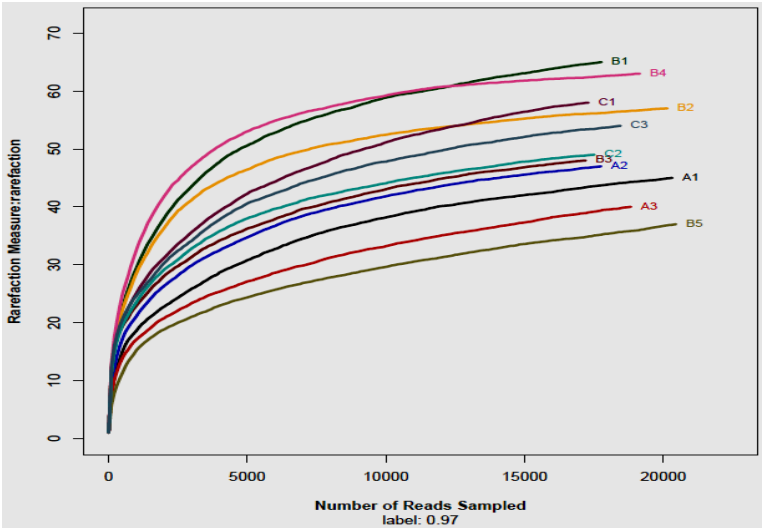

Fig. 2: Rarefaction analysis of the 11 milk samples. The curves were generated for 97% OUT levels. A1~A3 (1th parity), B1~B5 (2nd parity), and C1~C3 (3rd parity).

The results showed that Firmicutes was the predominant phylum in the current study. Other studies have also shown that Firmicutes and Tenericutes were the most dominant bacterial phyla detected in the milk samples or other organs of dairy cows with mastitis (Santos and Bicalho, 2011; Bhatt *et al*., 2012). Firmicutes was reported to be found in milk, the teat apex and uterus (Bhatt *et al*., 2012; Braem *et al*., 2012), and the results may be due to the prevalence of the Firmicutes phylum in a wide variety of habitats (Revilla-Guarinos *et al*., 2014). At the genus level, many previous studies suggested that *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Escherichia coli* were the most dominant bacteria that cause bovine mastitis because they are common pathogens that are hard to control (Bradley, 2002). Surprisingly, our results suggested that *Enterococcus* was the predominant bacterial group in the milk samples from the cows, and it was also detected in dairy goats (Martin-Platero *et al*., 2009). These results may be explained by the following reasons. First, the predominant mastitis-causing pathogen has changed rapidly due to many factors, such as climate change (Hogan and Larry, 2003). The high temperatures and relative humidity of the climate in Guangxi Province favors the growth of bacteria, and *Enterococcus* can survive in heat (Martin-Platero *et al*., 2009; Li *et al*., 2014). Under these circumstances, *Enterococcus* grew better than the other common mastitis-causing pathogens. Second, this farm used to apply penicillin, gentamicin, ceftiofur and streptomycin to treatment bovine mastitis. This antibiotic therapy influenced the growth of these common mastitis-causing bacteria, and they were restrained. In our study, *Staphylococcus* was inhibited by gentamicin, penicillin and streptomycin as in former studies (James, 2014). It was also confirmed that the long-term effects of antimicrobial treatment were effective against mastitis caused by *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* (Sandgren *et al*., 2008). These common pathogens were restrained while *Enterococcus* showed resistance to multiple antimicrobial drugs such as gentamicin, penicillin and streptomycin (Cortes *et al*., 2006). Furthermore, *Enterococcus* is a major environmental mastitis-causing pathogen that was found in the cow farm environment (Elhadidy and Zahran, 2014). Therefore, the results showed that *Enterococcus* was the predominant bacterial genus.

Tenericutes was the second dominant phylum that was detected in the milk samples. At the genus level, *Mycoplasma* was the predominant bacterial group. In a previous study, *Mycoplasma* was also detected in goats (Muhammad *et al*., 2016). In our study, the parity, management and antibiotic therapy were influenced by the diversity of *Mycoplasma*. First, the results showed that *Mycoplasma* was more abundant in the first and fifth parity compared to the third parity, which indicated that the cows were more sensitive to this pathogen in early age (first parity). In addition, as dairy cows age and organism degeneration increases, the function of the sphincter muscle that mediates the teat orifice is affected; when the function of the sphincter muscle degenerates, it becomes an entrance for mastitis-causing bacteria. Thus, *Mycoplasma* most likely enter through the teat orifice (Braem *et al*., 2012). Moreover, with increased parity, the immune response of cows is easily impaired. Once stressed, cows become infected with Mycoplasma easily (Aebi *et al*., 2012). Therefore, *Mycoplasma* was more abundant in the fifth parity. Second, M*ycoplasma* was also described as a common pathogen and is frequently present in the cattle population; larger herds have a higher risk of *Mycoplasma* infection. It was also demonstrated that *Mycoplasma* was transferred during milking time by fomites contaminated with the pathogen, such as milk, teat cup liners or other surfaces of the milking equipment (Punyapornwithaya *et al*., 2011; Pinho *et al*., 2013). Thus, management is another factor that influences *Mycoplasma* diversity. Third, this dairy farm used to apply penicillin, streptomycin, gentamicin and ceftiofur to treat and control mastitis. However, the results showed that this was not effective for controlling *Mycoplasma* as it is resistant to most antibiotics, including penicillin, streptomycin, gentamicin and ceftiofur (Schultz *et al*., 2012). Conse-quently, *Mycoplasma* was prevalent in the current study.

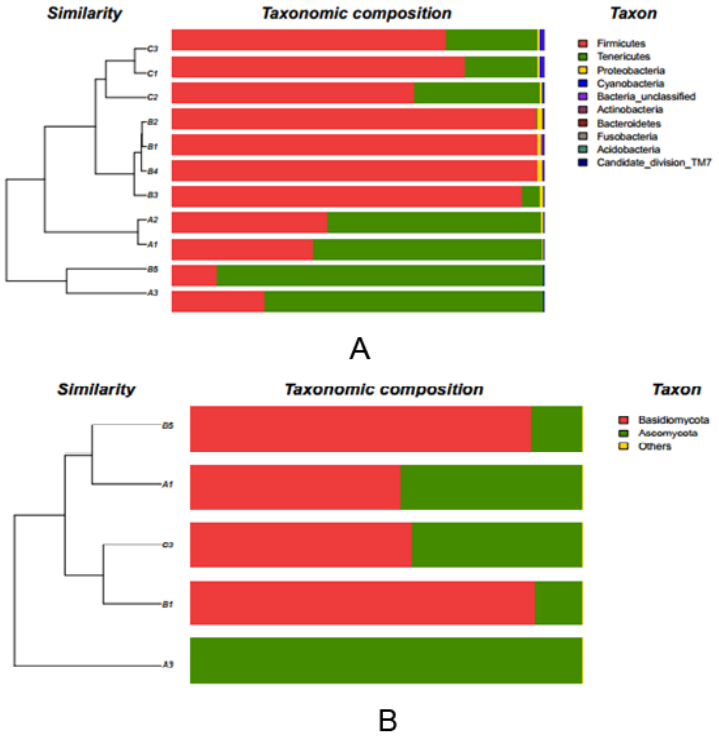

Fig. 3: H-cluster tree analysis and the taxonomic composition of bacteria (A) and fungi (B) in the 11 milk samples. Hierarchical dendrogram showing the bacterial distribution among the 11 samples. A1~A3 (1th parity), B1~B5 (2nd parity), and C1~C3 (3rd parity).

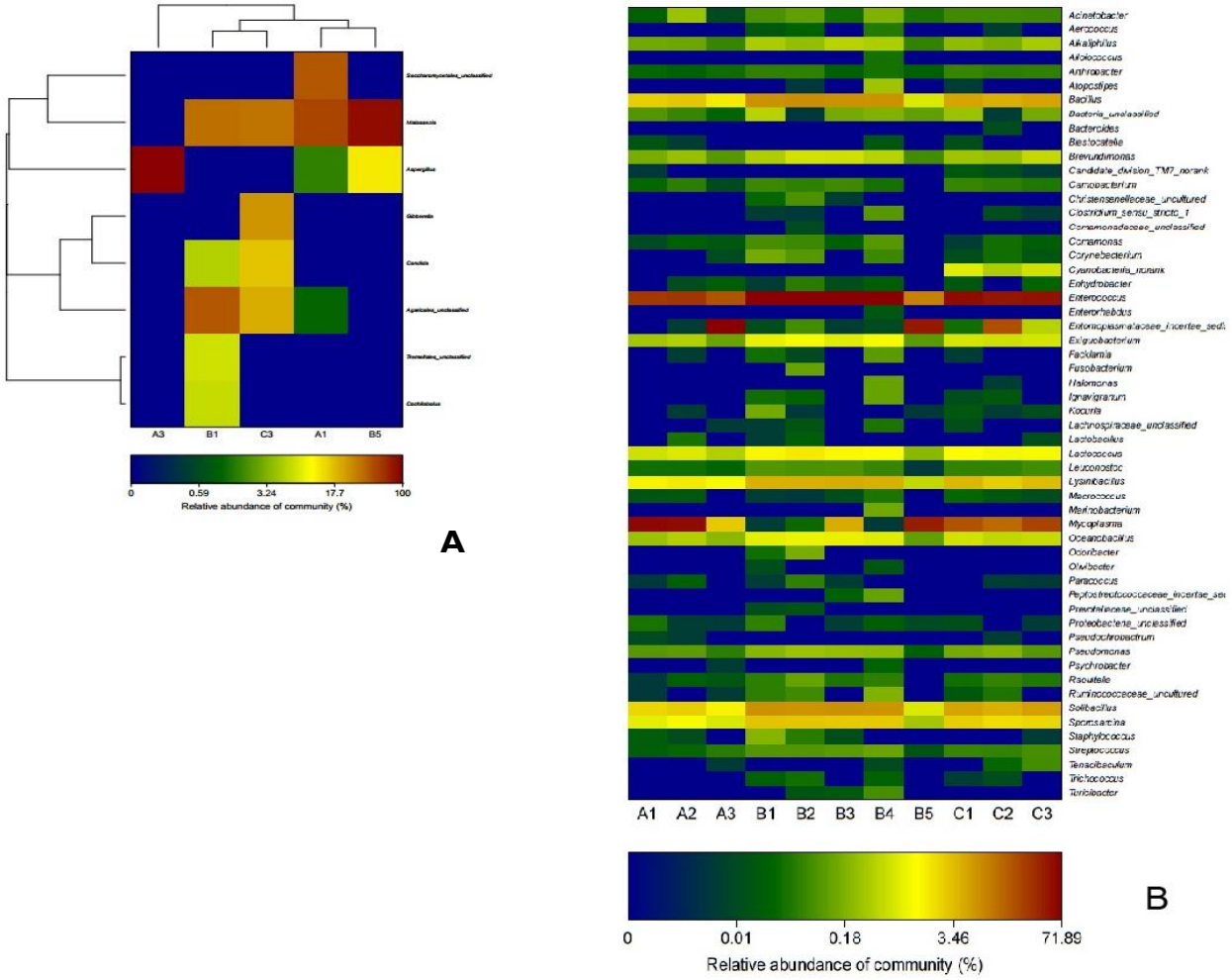

Fig. 4: Heatmap of the fungal distribution of the different communities (A). Heatmap of the bacterial distribution among the eleven samples at the genus level (B). The heatmap plot depicts the relative percentage of each bacterium in the community. The relative values for the bacterial community members are depicted by color intensity with the legend indicated at the bottom of the figure. A1~A3 (1th parity), B1~B5 (2nd parity), and C1~C3 (3rd parity).

Table 2: The parity, mastitis history and health condition three months after illness for the dairy cows. A1~A3 (1th parity), B1~B5 (2nd parity), and C1~C3 (3rd parity).

Sample ID	Parity (frequency)	History of mastitis (frequency)	Three months after the illness
A1	1	0	Stop milk
A2	1	0	Healing
A3	1	0	Repeated recurrence
B1	3	2	out
B2	3	1	Healing
B3	3	2	Healing
B4	3	2	Repeated recurrence
B5	3	3	Out
C1	5	2	Stop milk
C2	5	2	Healing
C3	5	4	Out

In this study, the results showed that the most abundant genus of fungi was *Malassezia*, followed by Agaricales-unclassified, Saccharomycetales, and *Candida*. This study was conducted in a subtropical area, Guangxi Province. Here, the annual mean temperature was 22-23°C, and the average relative humidity was 79.07%. These conditions benefit the growth of fungi such as Candida and Aspergillus. Previous studies proved that the high environmental temperatures (15-35°C) were an important factor for fungi mastitis (Zhou *et al*., 2013).

In this study, the most abundant phyla were Firmicutes and Tenericutes. At the genus level, the predominant bacterial groups were *Enterococcus* and *Mycoplasma*. These findings remind us that the mastitis-causing bacteria are changing, and identifying them through effective methods is necessary for prevention and treatment, especially antibiotic therapy.

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