RESEARCH ARTICLE

Application of high-throughput sequencing technology in the analysis of fungal community succession during the rice storage

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China is a leading producer and consumer of rice in the world. The rice storage conditions after the harvest affect the physiological and metabolic process and then the quality of the rice. This study investigated the succession of fungal communities in Northeastern japonica rice under different moisture conditions using simulated storage conditions. All rice samples were divided into two groups based on their moisture contents with the high moisture rice (HW) group from 14.50% - 15.50% and low moisture rice (LW) group from 13.50% - 14.50% under the storage conditions. The results showed that the moisture of two rice groups decreased in the first and second storage periods from 15.50% to 15.09% in HW group and from 14.21% - 13.59% in LW group, respectively, while the moisture of two rice groups remained stable in the third storage period. According to the species classification analysis using Operational Taxonomic Units (OTUs), the fungal communities of two rice groups underwent different succession changes. The fungal community of high-moisture or HW rice demonstrated obvious succession in each storage period. At the end of storage, *Papiliotrema aurea*, *Curvularia inaequalis*, *Neosetophoma samararum*, and *HannAella sinensis* were determined as the final dominant species. On the other hand, the fungal community of low-moisture or LW rice group showed obvious succession in the second storage with *Curvularia inaequalis*, *Hannaella sinensis*, and *Hannaella zeae* as the dominant species at the end of storage. The results of this study provided a basis for the establishment of an anti-mildew system for specific molds during rice storage.

Keywords: rice storage; fugus; microbial community structure; high-throughput sequencing.

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Introduction

The cultivation of rice has a long history [1]. As the world's leading producer and consumer of rice, the annual rice production in China has reached 212 million tons [2]. After harvest, rice needs to be stored to provide food for people during the non-harvest season or to cope with reduced production or starvation in the following years [3]. Given its substantial population, China must maintain food reserves to meet staple food demands [4]. Specifically, the post-pandemic era's fluctuations in food supply have placed significant pressure on countries and populations reliant on food imports [5]. Factors such as global economic disparities, climate change, and the ongoing urbanization process contribute to the necessity for China to sustain large-scale grain storage over extended periods [6, 7]. Rice served as the principal grain reserve in China is predominantly stored in its husked form, which complicates the monitoring of quality changes through conventional methods. The storage conditions were capable to accelerate or slowing

down the metabolic and physiological processes during rice storage.

Previous studies found that high temperature and high humidity (HT-HH) on the rice storage sites accelerated the metabolism of the harvested rice, and therefore, caused the quality changes of rice seeds [8]. Post-harvest rice typically contains 20% to 30% moisture, which is a necessity for timely drying to prevent mold growth. During the storage period, the temperature of the grain mass varies with the ambient conditions within the storage facility [7, 9]. Specifically, the surface and wall-adjacent areas of the grain mass are more susceptible to external environmental influences, constituting the active zones of grain temperature variation, whereas the central region remains relatively unaffected, forming a cold core [10-13].

This study used the newly harvested early Northeastern japonica rice in Northeast China as the research subjects. Two groups of rice were selected in this research including the high moisture rice (HW) group with the moisture content of 14.5% -15.5% and the low moisture rice (LW) group with the moisture content of 13.5% - 14.5%. All rice groups were stored for 18 months. During the period of rice storage, high-throughput sequencing technology was applied to analyze the succession changes of rice fungal communities at different storage periods with different initial moisture contents. This study would provide a basis for the establishment of an anti-mildew system for specific molds during rice storage.

Materials and methods

Sample collection

The newly harvested northeastern japonica rice samples were collected and stored in the No. 4 Tall Pingfang warehouse at the Mingshan National Grain Reserve in Benxi, Liaoning, China. The collected rice samples were dried naturally before being divided into two groups based on the measurements of moisture analyzer with the high moisture group (HW) showing the moisture content of 14.5% - 15.5% and low moisture group (LW) showing the moisture content of 13.5% - 14.5%. Under impurity less than or equal to 0.3%, in a simulated warehouse that was designed as 1.1 × 1.1 × 1.8 m^3 (Length × width × height), the simulated storage conditions that were determined according to the actual warehouse conditions and maintained *via* ventilation were set as 17℃ - 25°C, humidity 45% - 75%, and the height of the grain bulk surface of 1.2 m. A total of 18 months storage period was adopted with the regular sampling and testing being conducted during the storage period on March 11, 2020, September 18, 2020, March 26, 2021, and September 1, 2021, and the corresponding tested samples being named as HW/LW, HWS1/LWS1, HWS2/LWS2, and HWS3/LWS3, respectively. Upon sampling, the storage temperatures and humidities were also recorded based on real-time measurement, which were used to divide the storage period into the first, second, and third periods. 150 g of each rice sample was taken with 50 g samples were weighed and put into a sterile homogeneous bag to make 3 parallel samples and stored at 4°C.

Determination of moisture

The moisture content of each sample was determined following the direct drying method recommended by “National Food Safety Standard for the Determination of Moisture in Foods” (GB5009.3-2016).

Determination of fatty acid value

The fatty acid value (FAV) of the sample was determined following the instructions of “Determination of Fatty Acid Value of Grain Mill Products” (GB/T 15684-2015) [14]. The rice samples were hulled using JGMJ8098 hulling machine (Shanghai Jiading Grain and Oil Instrument Co. Ltd, Shanghai, China) and ground into powder using BLH-560KL mill (Zhejiang Bethlehem Apparatus Co., Ltd., Taizhou, Zhejiang, China). The fatty acids were extracted from the rice powder using anhydrous ethanol followed by titration using 0.05 mol/L KOH. Phenolphthalein solution was then applied as an

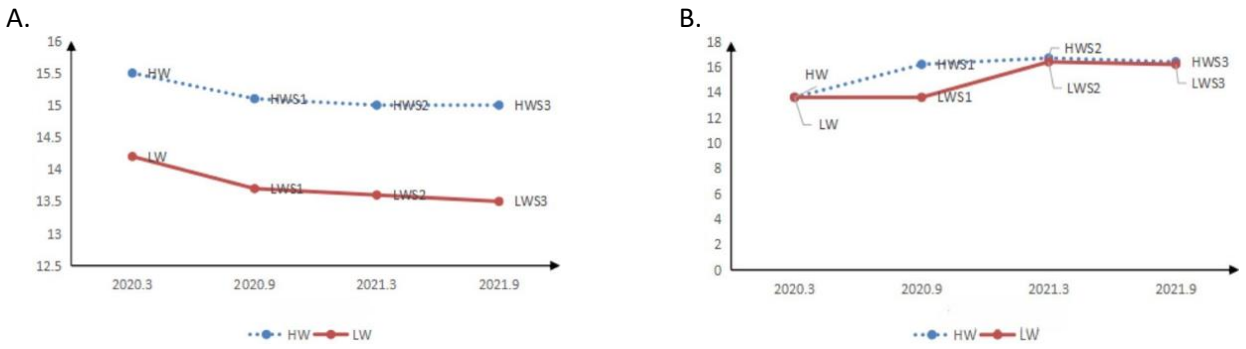

Figure 1. The moisture (A) and fatty acid values (B) in each group.

indicator. The FAV was determined as the milligrams of KOH required to neutralize the free fatty acids in 100 g of rice powder.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

A total of 25 g sample was mixed with 225 mL sterile water in a conical flask for 30 min followed by 10 to 1 dilution. The bacterial genomic DNAs were extracted and purified. PCR amplification was then carried out using Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions in 50 µL reaction system with forward and reverse primers of internal transcribed spacer-1 (ITS-1) as GGA AGT AAA AGT CGT AAC AAG G and GCT GCG TTC TTC ATC GAT GC, respectively. The standard fungus ITS-1 was about 250 bp and was purified through electrophoresis using AXYGEN Gel Extraction Kit (Axygen, Union City, CA, USA). The sequencing library was prepared using the TruSeq Nano DNA LT Library Prep Kit (QIAGEN, Hilden, Germany) and sequenced using Miseq-PE250 system (Illumina, San Diego, CA, USA).

Data analysis

FASTP (https://github.com/OpenGene/fastp) was employed to remove the adaptors and low-quality reads. QIIME2 (https://qiime2.org/) and Vsearch (http://vsearch-lab.org/cd-hit/) were applied for sequence denoising and operational taxonomic unit (OUT) clustering. QIIME2 phylogeny align-to-tree-mafft-fasttree and call mafft (https://github.com/voutcn/megahit) were used for multi-sequence alignment and mask the parts without phylogenetic information. Bacteria taxonomic and functional profiles were then obtained with the resulted gene sets being aligned to the National Center for Biotechnology Information (NCBI) non-redundant (NR) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). The microbiome diversity analysis including alpha and beta diversities were conducted and visualized using the vegan and ggplot2 packages in R (version 4.0.2) (https://www.r-project.org/). To comprehensively evaluate the Alpha diversity of the fungal flora in the samples, Chao1 and observed species indexes were used to characterize the richness of the sample fungal flora, while Shannon and Simpson indexes were used to characterize the diversity of each sample [15]. Pielou's evenness index was used to characterize uniformity [16], and Good's coverage index was used to characterize coverage [17].

Results and discussion

Changes in sample moisture and fatty acid values

The samples were collected at four sampling times with the storage conditions of temperature (℃)/moisture (%) as 16.2/52, 20.2/55, 16.5/69, and 23.3/71. The storage times were divided into the first, second, and third storage periods, which covered the times of March 11, 2020 to

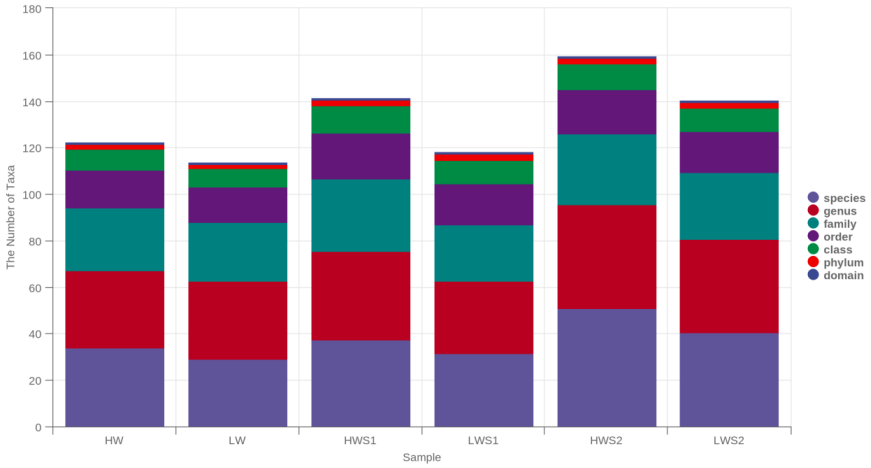

Figure 2. Taxonomic annotation. All samples annotated at the phylum level had shorter column order, while those annotated at the genus and species levels had longer column order.

September 18, 2020, September 18, 2020 to March 26, 2021, and March 26, 2021 to September 1, 2021, respectively. The results showed that the moisture contents of HW and LW rice groups decreased during the first and second storage periods and remained stable in the third storage period with the moisture contents being reduced from 15.5% to 15% and 14.2% to 13.6% for HW and LW groups, respectively (Figure 1A). The changing trends of the fatty acid values between two groups demonstrated obvious differences. The results showed that the fatty acid of HW group increased significantly during the first storage period from the initial 13.6 mg/100 g to 16.2 mg/100 g, while it became slower from 16.2 mg/100 g to 16.7 mg/100 g in the second storage period and stable as 16.6 mg/100 g in the third storage period. The fatty acid value of LW group maintained a stable trend in the first storage period as 13.6 mg/100 g to 13.7 mg/100 g and then increased sharply in the second storage period from 13.7 mg/100 g to 16.4 mg/100 g, while it stabilized again in the third storage period as 16.3 mg/100 g (Figure 1B). The changes in moisture and fatty acid values of both groups stabilized in the third storage period, indicating that the microecological environment of the fungal communities tended to stabilize in the two groups. Therefore, the rice samples of HW and LW groups in the original state (HW and LW), at the end of the first storage period (HWS1 and LWS1), and at the end of the second storage period (HWS2 and LWS2) were selected for metagenomic analysis and ITS-1 sequencing, which involved 18 rice samples.

Taxonomic annotation of fungi in rice

UNITE database (https://unite.ut.ee/) was used to identify the 18 rice samples at each taxonomic level. The results were plotted as a bar graph, which allowed intuitive comparison of differences in the number of OTUs and classification status in rice samples between different groups. The results showed that the fungal communities in rice with different moisture levels belonged to 2 phyla, 12 classes, 20 orders, 31 families, 44 genera, and 51 species (Figure 2).

Diversity of fungal community in rice

The coverage index of all 18 rice samples reached 0.99, indicating that the coverage of the sequencing results met the requirements. For Chao1, Simpson index, the diversity of HW group demonstrated a trend of first decrease and then increase with HWS2 > HW > HWS1, while LW group displayed a gradually increasing trend with LW < LWS1 < LWS2. The results suggested that HW rice had carried more types of fungi (HW > LW) than that in LW rice before entering storage period. However, after entering storage, this part of fungus displayed mass mortality due to the

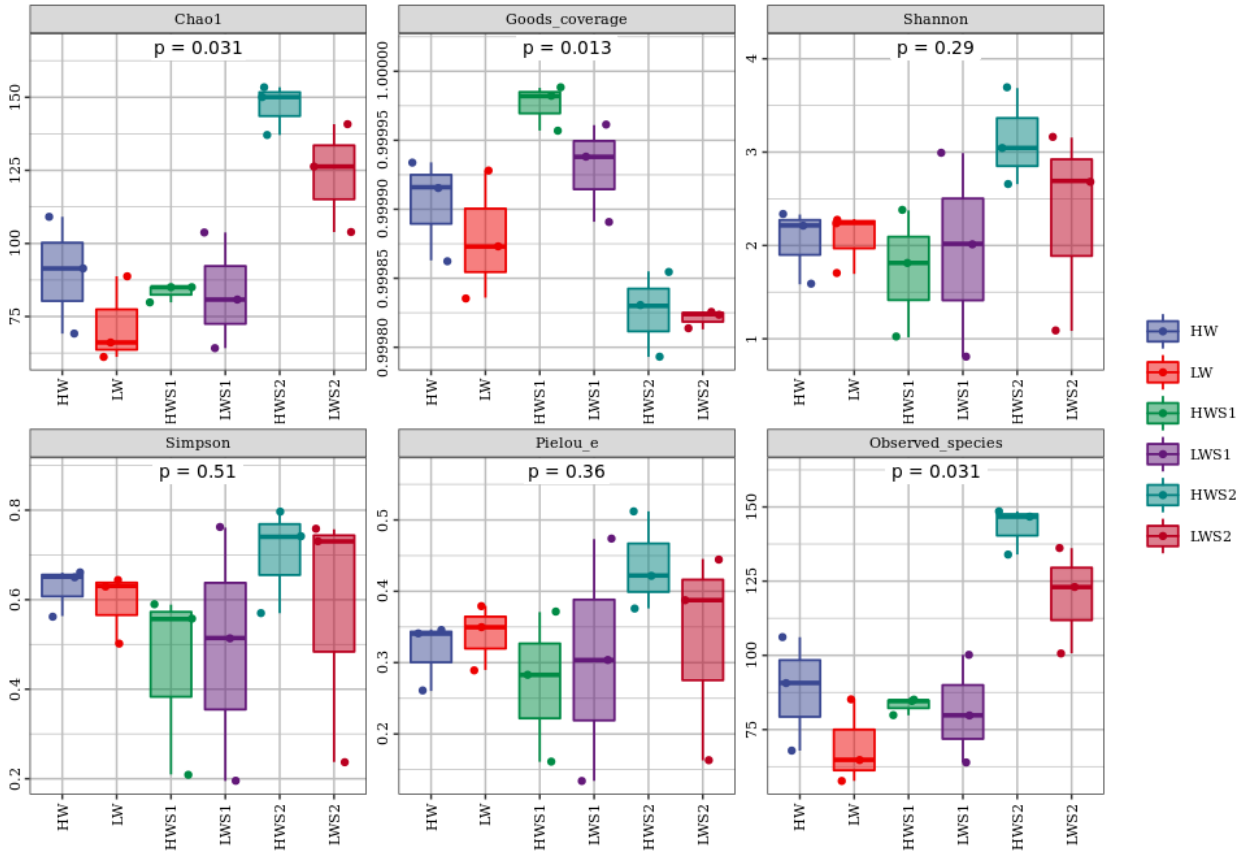

Figure 3. The box plots figures of samples.

restricted storage conditions, so the diversity index began to decline (HWS1 < HW). HW rice provided a suitable environment for the growth and reproduction of storage fungi. Therefore, during the extension of storage time, the fungal diversity index of HW rice started to increase (HWS2 > HW > HWS1). On the other hand, due to low water content, LW rice carried fewer types of fungi before entering storage. After entering storage, the storage fungi began multiplication. Therefore, along with the extension of storage time, the diversity index presented a gradually increasing trend (LW < LWS1 < LWS2) (Figure 3). The results indicated that the higher moisture content of the rice was more conducive to the survival of fungi.

The composition difference of fungi in rice at different classification levels

The diversity of fungal in each sample at different storage periods were classified into 5 phyla of *Ascomycota*, *Basidiomycota*, *Mortierellomyco*, *Glomeromycota Rozellomycota*. The number of *Basidiomycota* in HW increased first and then decreased during the remaining storage time with HW, HWS1, HWS2 as 53.2%, 74.6%, 55.8%, respectively. In LW, with the extension of storage time, *Basidiomycota* presented a continuous increasing trend with LW, LW1, LW2 as 44.6%, 73.9%, 85.4%, respectively (Figure 4). The structure of fungi in both groups in each storage period was classified and counted at the species level. Ten species with relatively high abundance were identified. Among them, *Magnaporthe grisea*, *Papiliotrema aurea*, *Curvularia inaequalis*, *Neosetophoma samararum*, *Hannaella sinensis* gradually increased in HW group during storage time (HW < HWS1 < HWS2), while *Filobasidium magnum*, *Magnaporthe grisea*, *Hannaella zeae* increased first and then decreased in the rest storage time (HW < HWS1 > HWS2). *Papiliotrema fuscus* first decreased and then increased during

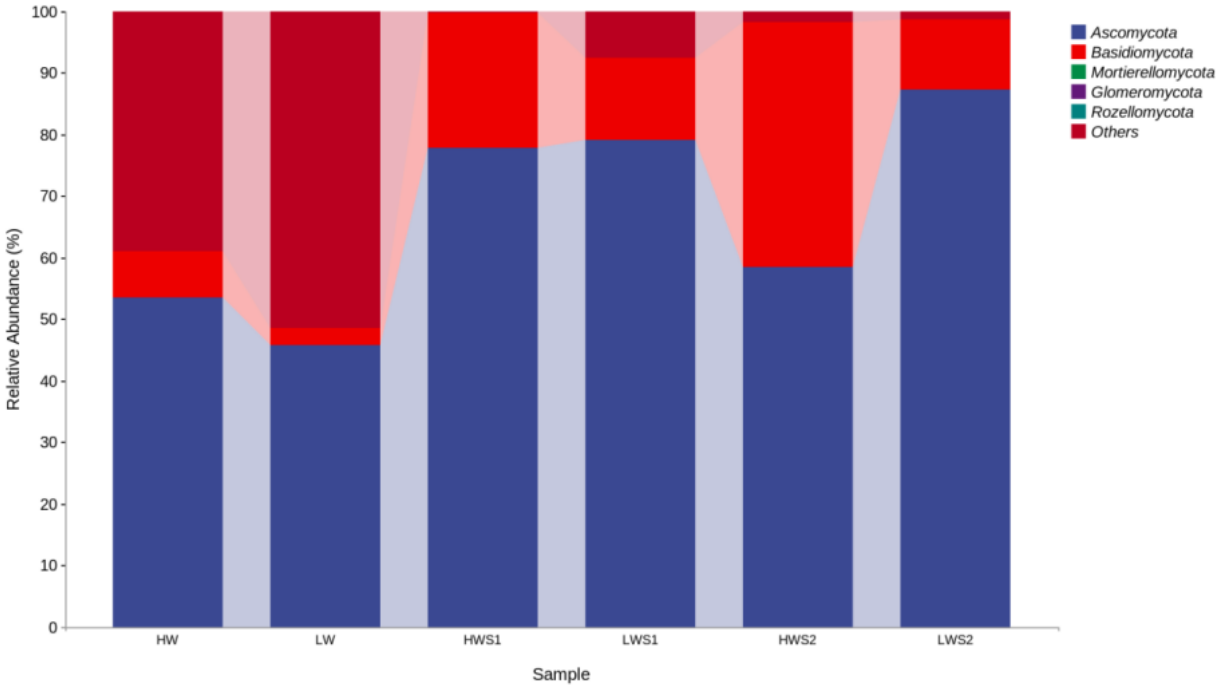

Figure 4. The fungi composition at the phylum level.

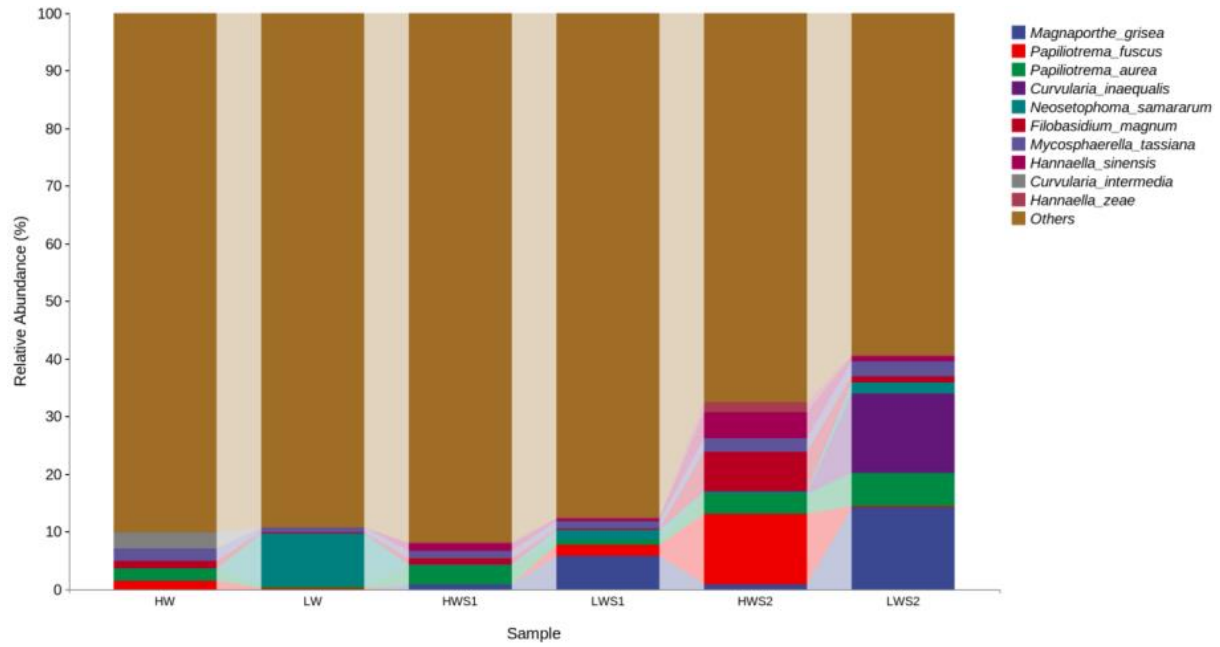

Figure 5. The fungi composition at the genus level.

storage time (HW > HWS1 < HWS2), and *Curvularia inaequalis* decreased to 0 after the first storage period, indicating that this species almost disappeared in HW rice. In the LW group, the relative abundance of *Curvularia inaequalis*, *Hannaella sinensis*, and *Hannaella zeae* showed 0 at the initial sampling, but increased significantly after two storage periods (LW < LWS1 < LWS2).

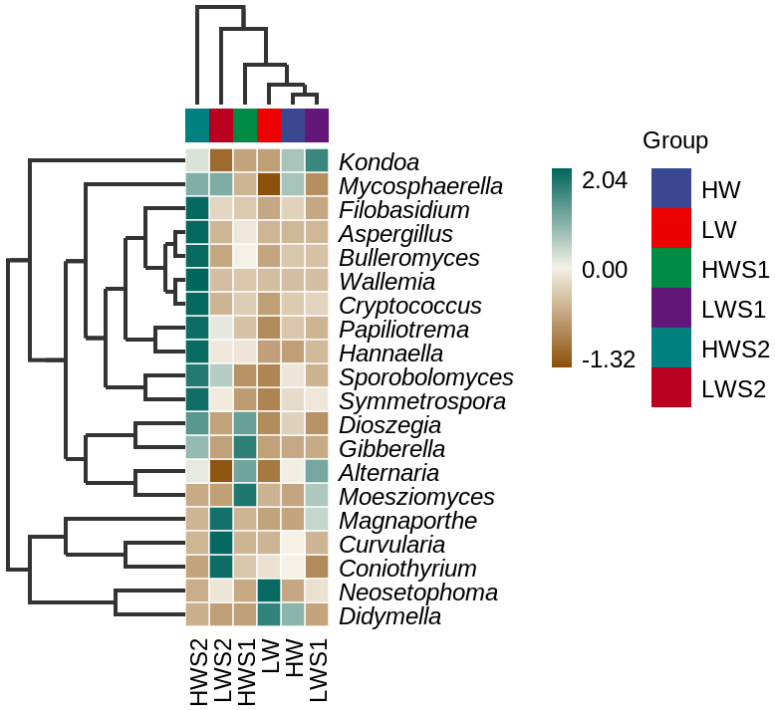

Figure 6. The heatmap of each sample.

Neosetophoma samararum, *Filobasidium magnum*, *Mycosphaerella tassiana*, and *Curvularia inaequalis* continually decreased (LW > LWS1 > LWS2), while *Magnaporthe grisea*, *Papiliotrema fuscus*, and *Papiliotrema aurea* increased first and then decreased (LW < LWS1 > LWS2) (Figure 5). Among them, *Magnaporthe grisea*, *Papiliotrema aurea*, *Curvularia inaequalis*, *Neosetophoma samararum*, and *Hannaella sinensis* accounted for a large proportion in HW group with continually increase. The results were consistent with the findings of Xu *et al*. that, under the storage conditions of 20℃ and 43% moisture, the main fungi of rice were *Neosetophoma samararum* and *Curvularia inaequalis*. Which both could cause a new type of leaf spot disease with symptoms similar to rice blast [18]. Therefore, by controlling the moisture content of rice before storage, it is possible to reduce the production of pathogenic fungi and guarantee the rice quality and safety.

Species composition and abundance heat map of fungi in rice

The heat map was used to compare the fungi species composition in each sample and illustrate species abundance distribution trend. The results showed that, in the HW group, with the extension of storage time, *Filobasidium*, *Aspergillus*, *Bulleromyces*, *Wallemia*, *Cryptococcus*, *Papiliotrema*, *Hannaella*, *Sporobolomyces*, and *Symmerospora* demonstrated increased relative abundance, while *Didymella* and *Kondoa* showed decreased relative abundance. In the LW group, with the extension of storage time, genera *Neosetophoma* and *Didymella* showed decreased relative abundance and reached 0 in the second storage period, while genera *Magnaporthe*, *Curvularia*, *Coniothyrium*, and *Neosetophoma* displayed increased relative abundance (Figure 6). Meanwhile, in the early storage period, HW and LW groups displayed similar fungi community composition with the dominant genera of *Neosetophoma* and *Didymella*. In the second storage period, the fungal community composition in HWS2 and LWS2 groups changed at the genus level, and the dominant genera

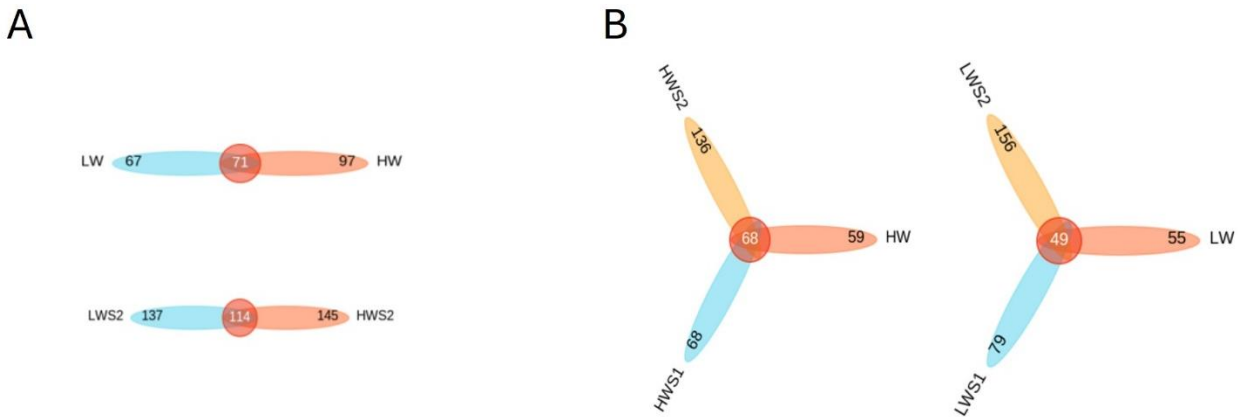

Figure 7. The Ven Plot. Different sample groups were denoted in different colors. The area where circles of different colors overlap was marked with the number of common OTUs

underwent obvious succession, which might be because the accumulation of rice metabolites produced mutual inhibition in the storage process [19, 20].

The difference of fungal communities in rice with different moisture content

Ven diagram was used for community analysis to investigate the common and unique species of different samples. Through the comparison of HW and LW rice groups, the results showed that the number of OTUs shared by HW and LW was 71, while HW had more characteristic OUT reaching 97. With the extension of storage time, HWS2 and LWS2 groups demonstrated that the number of OTUs shared by the two groups increased to 114 with the numbers of characteristic OTUs of the two groups increased to 137 and 145, respectively (Figure 7A). In the HW group storage, the samples in the three storage periods had 68 common OTUs. The longer the storage time, the more characteristic OTUs in the sample (HW < HWS1 < HWS2). In the LW group storage, the samples in the three storage periods had 49 common OTUs. As the storage time prolonged there were more characteristic OTUs in the sample (LW < LWS1 < LWS2) (Figure 7B). The results suggested that HW rice had higher fungal community richness than that of LW rice before storage. As storage progressed, HW rice still had higher fungal community richness than that in LW rice. Even for LW rice, the fungal community richness also increased during the storage period. High-throughput sequencing technology can access much information on fungal species and its abundance, but only some fungi could further grow and develop under storage conditions, producing an adverse effect on rice under certain conditions [21].

Beta diversity analysis

Principal component analysis (PCA) was carried out on HW and LW rice groups. The closer distance between the fungal community in rice with different moisture content and different storage periods suggested higher community similarity. The results showed that HW rice and LW rice were relatively close in the first storage period (HWS1 and LWS1), indicating small difference in fungal community diversity in rice samples. LW rice was close between the original state and the first storage period (LW and LWS1), indicating that the fungal community diversity of LW rice was relatively small during the first storage period. However, LW rice in the second storage period (LWS2) was far from that in other storage periods, indicating big difference in diversity of the fungal community. HW rice (HW, HWS1, HWS2) was relatively scattered during the three storage periods, indicating great species diversity of the fungal community in the rice samples (Figure 8). The results suggested that the fungal community diversity of LW rice had little

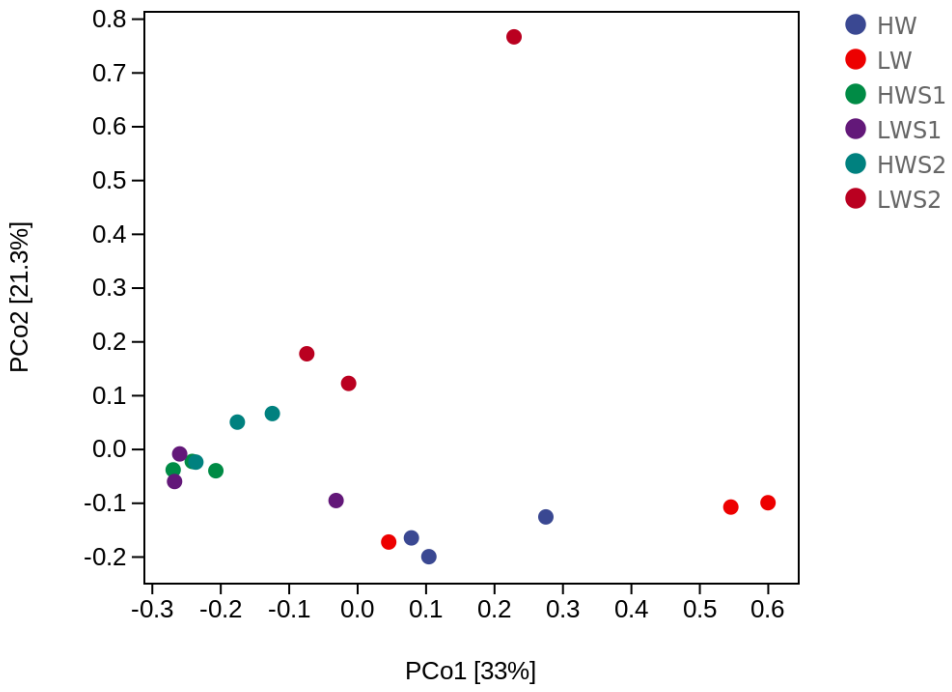

Figure 8. Principal component analysis in each sample.

change before the end of the first storage period, and then increased after the second storage period, while the fungal community diversity of HW rice increased in all storage periods, displaying obvious succession in the fungal community.

During the storage of rice, the microbial community changes are the result of the interactions between the microorganisms themselves and their environment including rice quality, environmental temperature, and humidity. The growth and metabolism differ between the same microorganisms in different environments or different microorganisms in the same environment [22]. This research investigated the changing trends of moisture and fatty acid values of rice with different initial moisture content and analyzed the succession changes of fungal communities in rice during different storage periods through high-throughput sequencing. The results confirmed that the moisture content of the rice affected the community succession changes during the storage period, and controlling the moisture content of the rice during storage was an important factor to guarantee the rice quality. Further, the results showed that the high-moisture rice and the low-moisture rice demonstrated a small difference in fungal community diversity during the first storage period, and a great difference during the second storage period, which indicated that the moisture content of the rice itself was a factor limiting the succession of fungal communities during the storage period.

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