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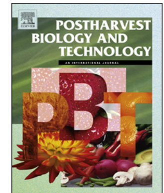
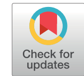


Compositional shifts in the surface fungal communities of apple fruits during cold storage



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ABSTRACT

The demands for fruit postharvest quality and safety improvement rely on a better understanding of the overall fungal communities during cold storage. Here, we characterized the superficial fungi of 18 apple samples before and after cold storage by sequencing internal transcribed spacer 1 (ITS1) sequences. High quality sequences (1,319,679) clustered into 512 operational taxonomic units (OTUs), representing 261 fungal species belonging to four phyla, 22 classes, 59 orders, 93 families, and 134 genera. The cold stored (CS) samples were harboured with 81,752 high-quality ITS reads and 265 OUTs per sample, which were higher than those of harvest point (HP) samples (averaged 64,879 ITS reads and 217 OTUs). Additionally, the CS samples obtained higher fungal richness, evenness and diversity than those of HP samples. Beta diversity analysis revealed significant fungi compositional differences between CS and HP samples. At the genus level, *Acremonium, Monascus, Archaeorhizomyces, Yarrowia* were highly detected in original HP samples, but reduced their abundance during cold storage; *Penicillium, Aspergillus, Mucor, Botrytis, Mycosphaerella, Rhodotorula* and *Wallemia* were significantly proliferated in CS samples. The increases level of *Penicillium, Aspergillus, Mucor,* and *Botrytis* during the apple storage, potentially suggest that the long-term stored apples contain more risks of fruit postharvest deterioration and patulin contamination. This study identified fungal communities on apples and their variations in cold storage, and provides a basis for further fruit fungi investigation.

1. Introduction

Apples have high acceptance as fresh fruit worldwide, and also used for processing juice, vinegar, wine, jam, apple crisp, etc. Based on FAO statistics (www/faostat.fao.org) for 2016, China is the largest apple producer, and devotes the greatest apple harvest area, accounting for 49.8% production (4.44×10^7 of 8.93×10^7 t) and 45.0% harvest area (2.38×10^6 of 5.29×10^6 hectare) worldwide. In China, apples are seasonally harvested (generally from September to November), and mostly preserved in cold condition for long-term marketing. In recent decades, the implement of cold preservation and transportation, extends apple postharvest storage, prolongs the shelf life, and promotes apple national and international trade significantly. However, the shifts of overall microbial communities during cold storage are only just beginning to be revealed (Graca et al., 2015; Tournas, 2005).

Previous studies indicated that apple surface is teeming with a wide variety of microorganisms, mainly fungi, that are closely associated with fruit postharvest deterioration (Magnani et al., 2007; Seow et al., 2012; Tadych et al., 2012). From a general point of view, the cold storage is generally considered slowing down the fruit ripening process, and delaying the growth of spoilage fungi (Morales et al., 2010; Prasanna et al., 2007). However, parts of phytopathogenic fungi are able to cause postharvest deterioration, affecting fruit safety and quality significantly. Using culture-based techniques, many investigators studied the fruit pathogenic fungi activities related to the postharvest infections, pathogenic confirmations, invasive biological mechanisms, and diseases control (Davis, 2014; Harteveld et al., 2014; Romanazzi et al., 2016). The long-term stored apple often decayed by the proliferation of related pathogens. Specifically, several species from *Aspergillus* and *Penicillium,* are the main fungi causing apple decay, and responsible for significantly economic losses (Fu et al., 2014; Morales et al., 2010). Furthermore, certain *Penicillium* species produce mycotoxin of patulin, a toxic fungal secondary metabolite, affecting quality of apple products, and causing consumer concern (Nie, 2017). To protect public health, many countries set the limitation for patulin (maximum levels ≤ 50 μg/kg) in apple products. Additionally, *Penicillium* sp. (Amiri and Bompeix, 2005) and *Aspergillus* sp. (Soliman et al., 2015), were reported enhanced their abundance in later stage of fruit

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storage. However, the fungi changes before and after cold storage are still unknown. Secondary, fruit also teem with a wide variety of endophytes. Several endophytes and bio-control agents protect fruits against postharvest biotic and abiotic stresses (Budziszewska et al., 2011; Supaphon et al., 2013). Besides, fruit surfaces also contain numerous unidentified fungi (Graca et al., 2015). Despite the improvement of technologies for preservation and postharvest disease control, until now, the complexities of fungal signatures on postharvest fruit are still unknown.

Traditionally, the culture-based techniques are only quantifying cultivable fungi belonging to specific taxonomic groups (< 5% of total microbes), which obstructs the understanding of overall fungal community. In recent years, next-generation sequencing (NGS) platforms (including SOLiD, Illumina and 454 sequencing) coupled with powerful database and user-friendly software are beneficial for the analysis of a complex microbial community at deeper, more comprehensive level (Kang et al., 2010; Kumari et al., 2016; Liu et al., 2016; Oliveri et al., 2017). To date, DNA NGS techniques have been successfully applied in microbial community analyses of various samples, such as soil (Li et al., 2017), air (Kumari et al., 2016), water (Liu et al., 2017), gut (Liu et al., 2016), leaf and root (Tarkowski and Vereecke, 2014), and fruit (Bokulich et al., 2014). Several studies recently reported the apple fruit microbial communities using NGS methods. Lopez-Velasco et al. reported the decrease in spinach phylloepiphytic bacteria communities following short-term refrigerated storage using pyrosequencing of 16S rRNA amplicons (Lopez-Velasco et al., 2011). Leff and Fierer studied the bacterial diversity of apple under organic and conventional farming practices using a 454 sequencing technique (Leff and Fierer, 2013). Glenn et al. studied the fungal and bacterial diversity on apple leaves and fruit using a 454 sequencing technique (Glenn et al., 2015). Abdelfattah et al. recently reported the fungal communities of organically and conventionally grown apples at the point-of-purchase using Illumina ITS1 sequencing (Abdelfattah et al., 2016a). However, little is known about the variations of overall fungal communities during fruit postharvest preservation. Exploration of NGS based fungal community and diversity on apple can provide insights into the fungi compositional changes during cold storage and provides a basis for further postharvest fungal analysis. Therefore, the changes of fungal communities during cold storage must be explored.

The objective of the present study was to evaluate the differences of fungal communities of apple surfaces before and after a period of cold storage using ITS1 sequencing. In our study, nine samples of harvest and stored samples were prepared and analysed respectively. Our results indicated remarkable fungal diversity on apple surfaces. Comparative analysis resulted that the stored samples contained more ITS sequences and OTUs. In addition, fungal composition varied significantly between stored and harvest point samples. Thirty-one genera were identified with statistically different abundance, and 15 of them were identified as key distinguished genera between two groups. Respectively, *Acremonium, Archaeorhizomyces* and *Yarrowia* were highly detected in harvest point samples, but reduced their abundance during cold storage. Twelve key genera, mainly of *Aspergillus, Botrytis, Mucor* and *Penicillium* were mainly proliferated in stored samples, suggesting that the long-term cold storage apples contain potential risk of fruit postharvest deteriorations and patulin contamination. This study compares the fungal diversity of apples at two typical points, and highlights the fungi compositional changes during cold storage to provide a basis for further investigation.

2. Materials and methods

2.1. Sample preparation and microbial DNA extraction

Nine apple samples were duplicate prepared (9 × 2), which were obtained from two orchards located in Xingcheng City, Liaoning Province, China, on October 10th, 2016. Fruit cultivating paper bags were placed on young fruits in mid-June and maintained on the fruit until sampling occurred. The typical two-layer fruit bag (Xiaolin Bag) was selected, which was comprised of a light impermeable out-layer (grizzly outside and black inside) and a red wax paper inner-layer. The first nine samples were analysed for fungal communities subsequently after harvest (harvest point samples, HP, n = 9) (Shen et al., 2018). The other samples covered with initial fruit bags, were placed in separated cardboard boxes, and then preserved in cold storage (0 ± 0.5 °C) without of any additional treatments until May 11th, 2017. Generally, the healthy (no visible disease) stored fruits were selected for further analysis (cold stored samples, CS, n = 9). The superficial total microbial DNA was collected by wiping or swabbing the apple surface (20 apples each sample) with cotton swabs (pre-moistened by sterile water). Sampling swabs were gathered and stored at −40 °C less than two weeks before microbial DNA extraction. Microbial genomic DNA was extracted from the swabs using the MoBio Power Water® DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of DNA extractions were measured by spectrophotometric analysis using NC 2000 (Thermo Scientific Fisher, Waltham, MA, USA), and by 1.0% agarose gel electrophoresis, respectively. The DNA extractions were stored at −80 °C until further use.

2.2. DNA amplification and ITS1 sequencing

The fungal rDNA ITS1 region was PCR-amplified according to processes described previously (Xia et al., 2016). The primers were ITS5F: 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS1R: 5′-GCTGCGTTCTTC ATCGATGC-3′. The PCR incubation system contains 5 U of DNA polymerase (New England Biolabs Inc. Ipswich, MA, USA), 15 pmol of both primers, 40 ng of template DNA, 2.5 mM of dNTP mixture, and 10 μL of 10× Buffer II in a total volume of 25 μL. The PCR amplification series was performed using an ABI 9600 instrument under the following conditions: initial denaturation at 94 °C for 4 min; 25 cycles of denaturation at 98 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s; and final elongation at 72 °C for 8 min. The amplicons were cleaned and purified using the Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) under the recommended instructions. The obtained ITS1 amplicons were sequenced on an Illumina MiSeq platform at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.3. Bioinformatics analysis

The pair-end reads from raw DNA sequences were conducted by using the FLASH software (Magoc and Salzberg, 2011). Data quality control and analysis were mainly conducted using the software package of QIIME (http://qiime.org/) (Caporaso et al., 2010). The high-quality sequences were obtained by discarding the low-quality sequences from raw data, including sequences shorter than 150 bp, with any ambiguous bases, containing mononucleotide repeats more than 6, Phred score below 25, or chimeric sequences (by QIIME). Then, the obtained high-quality sequences were assigned into operational taxonomic units (OTUs) at 97% similarity, by the QIIME-uclust OTU-picking workflow (Edgar, 2010). OTUs taxonomy were identified by BLAST searching of a representative sequence of each OTUs against the UNITE database using the best hit (Release 5.0, https://unite.ut.ee/) (Edgar, 2010). The original OUTs table was created, that contained the taxonomic classification for each OTU and the matrix of OTU corresponding abundance for each sample. To reduce the complexity for subsequent analyses, the OTUs table was simplified by removing the rare OTUs (≤0.001%) that contain equal or less than 13 sequences (Koljalg et al., 2013). The final modified OTUs table (Table S1) was rarefied and used for subsequent analyses.

The OTUs rank abundance curves and the rarefaction curves were plotted to explain both richness and evenness of fungi community (Heck et al., 1975). ACE and Simpson indexes were calculated to

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